The distribution, exposure dynamics and toxic significance of 42 polychlorinated biphenyls (including three coplanar congeners), in biota and sediment from the Western Basin of Lake Erie.

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THE DISTRIBUTION, EXPOSURE DYNAMICS AND TOXIC SIGNIFICANCE
OF 42 POLYCHLORINATED BIPHENYLS
(INCLUDING THREE COPLANAR CONGENERS),
IN BIOTA AND SEDIMENT FROM THE WESTERN BASIN OF LAKE ERIE

by

Susan Elizabeth Koslowski

A Thesis
submitted to the
Faculty of Graduate Studies and Research
through the Department of Biological Sciences
in Partial Fulfillment of the requirements
for the Degree of Master of Science at
the University of Windsor

Windsor, Ontario, Canada

1993

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ISBN 0-315-87367-1
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ABSTRACT

Polychlorinated biphenyls are ubiquitous pollutants which are associated with wasting syndrome, reproductive problems, teratogenicity and mortality in wildlife (Borgmann et al. 1990; Kubiak et al. 1989). There are 209 PCB congeners which differentially partition, bioaccumulate and degrade in the environment. It is important to analyze the exposure dynamics of individual PCBs, as congener toxicity and persistence varies considerably.

This study examined the dynamics and distribution of 42 congeners in sediment and biota from the Western Basin of Lake Erie. The non-ortho substituted congeners IUPAC #77, #126 and #169 were quantified, and their concentrations were examined in various trophic levels. The AHH induction potency of each coplanar PCB was determined using the AHH assay on rat (H4IIE), mouse (Hepa Clone 9) and human (Hep G2) cell lines.

The PCB dynamics in the food web sampled were more complex than predicted by thermodynamic partitioning. Tetrachlorinated biphenyls were found to dominate lower trophic level samples; whereas, predatory animals exhibited much higher levels of hexa- and heptachlorinated biphenyls. The composition of PCB congeners was different for each trophic level, while concentrations of total PCB (equal to the sum of 42 congeners) increased with increasing trophic level. There were few differences in the distribution of ortho-substituted PCBs among species' tissues.
Coplanar PCBs were found in Lake Erie species. Residues of congener #126 and #169 were detected in herring gull eggs and in benthic organisms only, whereas #77 was present in all samples. There were significant differences in the partitioning of coplanar PCBs between muscle, liver and egg tissues for all fish species sampled. The proportion of total PCB congeners that were coplanar decreased with increasing trophic level.

The AHH assay indicated that congener #126 induced Hepa Clone 9 mouse cells more than #77 or #169, and slightly less than TCDD. The H4IE and Hep G2 cells did not give consistent results; however, the coplanar PCBs did induce AHH activity in both cell lines. Experiments with tissue extracts gave very low levels of enzyme activity, while chemical combinations induced lower activity levels than anticipated. This may have been the result of competitive inhibition by less potent inducers such as congener #77.
To Arl, Welt and Greg
ACKNOWLEDGEMENTS

This Master of Science degree owes much to the individuals I worked with at the University of Windsor.

I must thank my two supervisors, Doug Hassner and Michael Dufresne, for their endless patience and good humour as I moved countries, married and pursued additional education. Their flexibility has been greatly appreciated! I'd like to thank Lynda Corkum and Jan Ciborowski for opening their home and labs to me, and for their invaluable advice on research and life. Ron Russell was my 'unofficial' supervisor and sounding board without whose guidance I would have been lost. A warm thank you to Elizabeth Hanes, Cynthia Lokker, Heather Morrison and Mike Weis for supplying lodging during my sojourns in Windsor. Ana Pascual-Dosescu was a source of strength and optimism during our 'cellular trials and tribulations', in addition to being a terrific lab assistant. Also, thanks to Kathy Nicholson for expert boat handling, Todd Leadley for graphics help, and Rodica, Gabriella, David and Ella for their shared expertise and meticulous work in the organics lab. Much appreciation goes to Dr. Okey at the University of Toronto for his generosity and advice.

Finally I would like to thank my family and Greg for their constant encouragement, lengthy phone calls and financial support. I would not have completed this degree without their insistence.
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CHAPTER ONE
GENERAL INTRODUCTION

The Great Lakes are an irreplaceable natural resource, which represent 20% of the world's freshwater supply. The lakes, described as 'sweetwater seas' by European explorers, support widespread industry, provide drinking water for millions, and sustain valuable commercial and sport fisheries. The basin is home to 35,000,000 people, as well as 476 known chemical pollutants (Moriarity 1988). This estimate of the number of chemicals is surprisingly conservative, given that the Organization for Economic Cooperation and Development (OECD, 1986) predicts that 1000 new substances enter daily use globally each year. These add to the 60,000 anthropogenic products already in commercial use; however, none of these estimates account for the unintentional formation of chemical by-products.

The degradation of the Great Lakes began with their settlement, and has escalated with increased urbanization and economic growth. The past 200 years of basin history have been punctuated with environmental crises, which have included overfishing, loss of fish habitat, and epidemics of cholera and typhoid due to the contamination of drinking water with raw sewage. Species invasions became prevalent at the turn of the century with the appearance of sea lamprey (*Petromyzon marinus*), and alewife (*Pomolobus pseudoharengus*); they have occurred more recently with the widespread distribution of the zebra mussel (*Dreissena polymorpha*). The accelerated eutrophication of Lake Erie, and the contamination of the ecosystem with DDT and mercury were also well-publicized
situations which illustrated the decline of a once pristine ecosystem. The disposal of xenobiotics in the lakes, has left wildlife and basin residents with a sobering legacy.

The 1987 version of the 1978 Great Lakes Water Quality Agreement between Canada and the United States demanded an 'ecosystem approach' to basin problems. The agreement was based upon a philosophy of "zero discharge" for persistent toxic substances, but only implemented policy that called for 'virtual elimination' of discharges of these substances. Unfortunately, the Great Lakes are not a closed system, but receive pollutant input from numerous sources, which include the atmosphere, the watershed, groundwater leachate, as well as direct discharge. Additionally, the hydraulic retention time of the lakes is large, and only one percent of the total volume is renewed each year. Any persistent substance added to the lakes will remain for a long time.

Industrial pollutants such as insecticides, pesticides, petroleum products, and organochlorines are of environmental concern because many are not easily degraded. These substances persist in the environment, and due to their hydrophobicity, accumulate to high concentrations in biota. An understanding of the exposure and effects of hydrophobic chemicals requires investigation of the processes which control their partitioning in the environment.

The concept of fugacity was developed to help explain the dynamic activity of contaminants in ecosystems (Mackay 1979). Fugacity uses thermodynamics to describe the movement of chemicals among discrete 'phases' or environmental compartments. It describes the 'escaping tendency' of a chemical substance from a phase, and has units of pressure. Environmental phases are characterized by their capacity (z) to hold a
chemical. When $z$ is small for an environmental compartment, only a small amount of a chemical substance will remain in that compartment. Hydrophobic contaminants accumulate in phases where $z$ (the fugacity capacity) is large, or where a relatively high concentration of contaminant is required to exert an appreciable escaping tendency. Such phases are usually high in organic carbon, and include sediment, dissolved organic matter, suspended particulate matter, plankton, and animal lipid.

Pollutants partition among environmental phases until equifugacity or the equivalent tendency to escape from each phase is reached. This equilibrium among compartments does not extend to contaminant concentrations, which differ according to the capacity of each phase to retain the contaminant. The relationship between fugacity and concentration can be expressed by:

$$f = c/z,$$

where $c =$ concentration of chemical in the phase and $z =$ fugacity capacity of the phase.

The accumulation of hydrophobic substances in biota at levels which exceed those in the environment is thus based upon chemical partitioning governed by fugacity, and not concentration gradients.

An important parameter for assessing the environmental behaviour of hydrophobic chemicals is the octanol-water partition coefficient ($K_{ow}$). $K_{ow}$ measures the partitioning of contaminants between octanol, a synthetic surrogate for animal lipid, and water. Log $K_{ow}$ values for PCBs range from 4.09 for biphenyl, to 8.18 for decachlorobiphenyl (Hawker and Connell 1988). The log $K_{ow}$ of individual compounds is correlated with the magnitude and rate of chemical uptake by aquatic organisms (Russell and Gobas 1989). Generally the rate of uptake for hydrophobic contaminants varies linearly with log $K_{ow}$,
however, chemicals with high log $K_{ow}$'s partition differently than predicted by this relationship (Gobas et al. 1989).

The uptake and storage of contaminants in aquatic animals is termed bioaccumulation. When pollutants are taken up directly from water, the process is termed bioconcentration. Ingestion of contaminated food, and chemical enrichment through higher trophic levels is referred to as biomagnification. Together these two uptake processes manifest in elevated contaminant body burdens (bioaccumulation), which act upon individuals to cause ecosystem-wide effects.

Polychlorinated biphenyls are one group of organochlorines found in the Great Lakes. These pollutants are not limited to the basin, but are found throughout the global ecosystem (Tanabe et al. 1987). Two hundred and nine different PCBs exist, and are known as congeners. These contain varying numbers of chlorine atoms substituted in a biphenyl ring, and are named according to the International Union of Pure and Applied Chemistry (IUPAC) standard notation (Ballschmiter and Zell 1980), (Figure 1.1). PCBs with the same number of chlorine atoms are given a common numerical prefix, such as 'tri-chlorinated biphenyl' for molecules substituted with three chlorines. The numbered positions on the biphenyl ring are grouped into three locational categories named ortho, meta and para. The location of chlorine atoms effects PCB configuration. Non-ortho-substituted biphenyls are quite planar, which alters their exposure dynamics relative to ortho-substituted congeners (Norstrom 1989).

PCBs were first manufactured in 1881, and were valued for their molecular stability, inertness, and heat capacity. Industrial applications of PCBs included their use
Figure 1.1  Polychlorinated biphenyl molecule with IUPAC numbering, and ortho, meta and para positions indicated.
PCB

ORTHO POSITIONS ARE: 2 2' 6 6'

META POSITIONS ARE: 3 3' 5 5'

PARA POSITIONS ARE: 4 4'
as hydraulic fluids, heat transfer fluids, plasticizers and organic diluents (Moore and Walker 1991; Safe 1989). Great Lakes water was initially contaminated with PCBs through accidental spills and the deliberate disposal of industrial waste. Today, landfill leachate, contaminated sediment, and atmospheric deposition are the main sources of PCBs (Camanzo et al. 1987).

After widespread contamination was documented in the late 1960s (Jensen 1969), PCB production decreased, and ultimately the manufacture, import and use of PCBs was curtailed in the United States in 1971, and in Canada in 1977 (Moore and Walker 1991). Currently, Canadian regulations restrict the use of PCBs to closed capacitors manufactured prior to 1980 (Strachan 1988); however, several countries including Japan and the Federation of Independent States still manufacture and use PCBs (Tanabe 1988).

PCBs are relatively insoluble in water and their solubility decreases with increasing chlorination of the biphenyl molecule. PCBs generally have low vapor pressures, and volatility is inversely related to the degree of chlorination (Shiu and Mackay 1986). Despite these physical characteristics, the Great Lakes and other bodies of water are contaminated with PCBs, and the atmosphere has been shown to be a "mobile environmental reservoir" of these pollutants (Ward 1986-1987). Wet and dry deposition are significant routes by which PCBs enter fresh water and marine ecosystems, despite the complex dynamics involved in the transfer of PCBs across the air-water interface. Ward (1986-1987) has shown that approximately ten times more PCB partitions into the aqueous phase than is predicted by equifugacity, because of chemical adsorption on dissolved and suspended particulate matter. The molecular stability which
made PCBs so attractive for industrial use, also made PCBs persistent and tenacious pollutants. Chlorinated biphenyls undergo some photochemical degradation; however, this process eliminates only an estimated 10 to 1000 g PCB/km² annually. Small concentrations of PCBs (0.01 to 500 μg/kg) stimulate bacterial growth by acting as carbon and energy sources; however, large concentrations of chlorinated biphenyls are toxic to microorganisms (Ward 1986-1987). Thermal degradation will break down large amounts of PCBs if pyrolysis occurs above 700°C. Temperatures lower than this will produce materials considered to be more dangerous than PCBs. Given that the cumulative world production of PCBs as of 1988 was estimated to be 1.2 × 10⁶ tonnes (Tanabe 1988), it is apparent that the only effective way to remove PCBs is through incineration. Regrettably, this process is expensive and largely inaccessible. Tanabe (1988) suggested that 31% of the total PCB produced had already been released into the environment, and that only 4% had been incinerated. More than double the quantity of escaped PCBs can be found in old electrical equipment, or in various forms of storage. Initial declines in aqueous PCB concentrations occurred primarily in heavily polluted areas. These concentrations have since stabilized, but levels have increased in areas where they were previously low or non-detectable (Borgmann and Whittle 1983). The future of PCB pollution is a long one, and contamination of the environment is likely to increase as stored PCB is mobilized.

PCB contamination is ubiquitous and far-reaching. Congeners have been documented in breast milk from Ontario, Quebec, Europe, and the Arctic at levels which exceed the Accepted Daily Intake of 0.1-10 pg/kg body weight/day (Dewailly et al 1991;
Krauthacker 1991; Galetin-Smith et al. 1990; Dewailly et al. 1989). PCBs were also detected in common fatty foods, such as beef, eggs, butter and cheese (Mes and Weber 1989). Elevated levels of PCBs have been found in numerous species of fish-eating birds (Borlakoglu et al. 1990; Kubiak et al. 1989), and fish (Williams et al. 1992; Gagnon et al. 1990), as well as in marine and terrestrial mammals (Kannan et al. 1989; Boon et al. 1987). These persistent organochlorines have been linked to cancer, teratogenesis, reproductive problems, immunotoxic responses, wasting syndrome, liver damage, and mortality (Borgmann et al. 1990; Kimbrough 1985).

The 209 PCB congeners differ with respect to solubility, volatility, persistence and toxicity. Each congener partitions differently among environmental phases which results in PCB residue distributions that are temporally and spatially diverse. Of greater concern is the relative risk posed by certain congeners. Depending on their structure congeners may be benign or toxic; however, unless the specific composition of congeners in samples is determined, the occurrence and abundance of the hazardous PCBs congeners remains unknown. Until recently analyses of PCBs in environmental samples rarely identified or measured individual congeners. Instead sample chromatograms were matched with similar chromatograms of Aroclor standards, and this rough matching then estimated the total PCB concentration in the sample (Swackhammer and Armstrong 1988). The Canadian government currently publishes PCB consumption and exposure guidelines as well as health advisories based upon total PCB content. This approach may well underestimate the hazard associated with PCB exposure should the more toxic congeners be abundant or enriched in samples. It is evident that some knowledge of the
partitioning behaviour and occurrence of individual congeners in the environment would provide valuable insight into their environmental transport, fate and health risk. Is ‘total PCB’ a useful measurement and does it adequately assess the hazards associated with PCB pollution, or does its relevance depend upon the false assumption that all congeners have similar exposure dynamics and toxicities? Additionally, what are the current levels of PCBs in the environment and in wildlife; are there detectable concentrations of toxic congeners, or have these declined to levels which are not hazardous? Before meaningful exposure guidelines may be issued, and prior to the development of environmental quality objectives for PCBs, it is important that the relative levels and distributions of PCBs including the more toxic congeners are known.

Environmental hazard assessment of PCBs requires exposure, accumulation, and effect data. This study examined the levels of PCBs in biota and sediment from the western basin of Lake Erie to investigate what regulates the exposure dynamics of PCBs. Chapter Two quantifies the exposure of Lake Erie fish and wildlife to total PCBs, and examines the pattern of PCB bioaccumulation in various species. Chapter Three presents the exposure dynamics and distribution of coplanar PCBs, which are thought to be responsible for many of the toxic effects produced by exposure to PCBs. In Chapter Four, preliminary work investigating the interaction between coplanar PCBs and their biological targets is presented. This research addressed the effect of individual congeners and congener combinations on human, rat and mouse cell cultures.
CHAPTER TWO

The Food Web Enrichment and Distribution of 39 Polychlorinated Biphenyls in the Western Basin of Lake Erie

Introduction

'Polychlorinated biphenyl' is the generic name given to 209 possible congeners, which are produced by the chlorination of a biphenyl molecule (Moore and Walker 1991; Safe 1989) (Figure 1.1). Commercial products such as Aroclors and Kaneclores (American and Japanese trademarks), range from 10% to 64% by weight, chlorine. The production of such PCB mixtures is an inexact process. Two batches of Aroclor 1248 will both contain 48% by weight, chlorine; however, they will differ with respect to the specific composition of congeners (Shiu and Mackay 1986; Oliver and Niimi 1983). The number and location of chlorine atoms on the biphenyl molecule determine the physicochemical properties of individual PCBs (Shaw and Connell 1984). It is accepted in the literature that congeners tend to increase in hydrophobicity and log K_{ow} with increasing chlorination, but decrease in volatility and in rates of uptake and biodegradation (Parsons et al. 1991). The activity of a given PCB congener following its release into the environment, is closely related to that congener's molecular structure. The pattern and amount of chlorine substitution also effect the biphenyl's bond angles, size, and hydrophobicity, which in turn influence congener kinetics, stability and toxicity. The science of predicting contaminant function from structure involves Quantitative Structure Activity Relationships (QSARs), which are essentially mathematical models that forecast the 'activity' and fate of classes of contaminants based upon their molecular structure.
While QSAR modelling has made general predictions possible, much remains poorly understood about the dynamics of PCBs in the environment.

Upon entering the aquatic ecosystem, PCBs partition among water, air, sediment, particulate matter, and the biota (Mackay 1989). Two models are often used to quantify the exposure dynamics of chemicals in aquatic ecosystems. The thermodynamic or fugacity approach to contaminant transport and fate predicts that PCBs will passively partition among ecosystem compartments until equifugacity is reached (Mackay 1989). At equilibrium, the fugacity of all phases, including animal lipid, will be equal; however, in aquatic ecosystems there are often differences between concentrations of PCB in lipid, particularly when species differ in terms of resource use and trophic level (Thomann 1992; Hebert 1991). The bioaccumulation of PCBs in aquatic biota is the result of some thermodynamic partitioning; however, it is apparent that certain aquatic animals do not reach equifugacity with their environment, and so it becomes important to investigate the processes which regulate biotic uptake and storage of PCBs.

The bioaccumulation of contaminants in fish is dependent upon bioavailability, rates of uptake and elimination, and metabolism. PCBs that are bioavailable are freely dissolved in the aqueous phase, and are available for transfer through the gill surface. This chemical uptake from the gills is known as 'bioconcentration' and is governed by thermodynamic partitioning. Fish may also absorb contaminants from their food and it is this process, known as biomagnification, that results in elevated body burdens. The food-web model postulates that as digestion occurs, the fugacity of the ingested food increases, and there is a net uptake of contaminant from the gastrointestinal tract (Clark
et al. 1990; Connolly and Pederson 1988). Each successive trophic level feeds on more contaminated prey and so organism chemical body burdens increase with increasing feeding levels.

The relative importance of bioconcentration versus biomagnification varies with individual congener characteristics, and with the degree of contamination of the biota. When a fish reaches higher chemical concentrations than the surrounding water there is a tendency for the fish to lose some chemical by diffusion through the gills, often enough such that there is no net uptake via the gills and biomagnification becomes the dominant process. In contrast, when fish are relatively uncontaminated there is chemical uptake from both the gills and the G.I. tract (Thomann 1992; Clark et al. 1990). Thus there is always exposure to chemicals via the gills but this exposure may not always result in uptake. As log $K_{ow}$ increases more PCBs become sorbed to particulates, and as the bioavailable fraction of chemical decreases so does the amount which may be bioconcentrated. Gill uptake of congeners with log $K_{ow}$s greater than 7 is small and, once again, biomagnification becomes the dominant exposure route (Clark et al. 1990).

Thomann (1981) compared the PCB residues in field samples of fish which experienced contaminant flux from food and water with residues in fish exposed in the laboratory to PCB through water alone. There were significantly greater PCB body burdens in field samples, which prompted the conclusion that in top predators most of the observed lipid residues could be attributed to the food-chain transfer of PCBs from contaminated prey. This finding was reiterated in a later study, where an age-dependent food chain model was used to describe PCB dynamics in the Lake Michigan food web.
(Thomann and Connolly 1984). Studies of natural aquatic communities which differed with respect to food chain length, revealed significantly elevated contaminant residues in lake trout when intermediate trophic species were present (Rasmussen et al. 1990). In a Lake Michigan food chain, PCB concentration and chlorine content were also found to increase with increasing trophic level, from plankton to sculpins (Evans et al. 1991).

As indicated in Chapter One, PCB congeners are chemically heterogeneous and will differentially bioaccumulate, biodegrade, and partition when released into the environment. Research suggests that bioaccumulation is dependent upon molecular stereochemistry, which influences PCB adsorption on and passage through biological membranes, and $K_{ow}$, which determines the partitioning of these congeners between aqueous and nonpolar phases (Parsons et al. 1991; Shaw and Connell 1984). Extreme hydrophobicity reduces the total contaminant flux from water to fish due to increased adsorption to particulate and dissolved organic material, and decreased concentrations of bioavailable PCBs (Oliver and Niimi 1985). Studies conducted in the St. Lawrence estuary on various aquatic organisms, indicated that the bioaccumulation of individual congeners varied among species, and was dependent upon molecular structure (Gagnon et al. 1990). This selective accumulation of congeners necessarily alters the PCB composition in biota relative to their environment.

Biodegradation of PCBs usually involves metabolism of the parent compound into a more water soluble form. Species differ with respect to their metabolic capacity for detoxifying organochlorines (Boon et al. 1987). Fish and invertebrates do not appear able to metabolize higher chlorinated congeners, and so the equilibrium partitioning of
PCBs between body lipids, blood and water becomes the most important factor affecting PCB kinetics in these animals (Bruggeman et al. 1981). The literature indicates that PCB elimination rates are related to stereochemistry and the degree of chlorination. Stalling et al. (1979) found that di- and trichlorinated congeners with varying numbers of ortho-substituted chlorines exhibited significantly different rates of elimination in rainbow trout (Salmo gairdneri). Similar results were recorded by Oliver and Niimi (1983) who found that levels of lower chlorinated congeners decreased faster in trout than did levels of highly chlorinated congeners. This study also demonstrated that congeners with vicinal hydrogen substitution or non-ortho chlorine substitution had significantly shorter half lives than congeners lacking such substitution patterns. Similarly Bruggeman et al. (1981) found that PCB elimination rates in goldfish (Carassius auratus) were variable and that chlorine substitution in the para positions slowed rates more than ortho substitution did. Research involving several species revealed markedly different congener residues in mammalian tissue when compared to fish tissue. This may relate to the exposure history of the species or to their metabolic capability (Boon et al. 1987 and Norstrom). These studies indicate that the composition of PCBs in animal tissue varies significantly among species, and does not resemble the original commercial mixtures that were released into the environment. The observed variability in bioaccumulation, elimination and metabolism of individual PCBs, suggests that it is important to look at the distribution of specific congeners when assessing organochlorine pollution.

The analysis of PCB residues is complicated by the large number of potential congeners, as well as by analytical handicaps such as the tendency of congeners to co-
elute, and difficulty in accurately interpreting chromatograms. Conventional analyses report environmental levels of PCBs in terms of total PCB concentration or Aroclor equivalents. These two measures are often derived from the concentration of PCB #138 (a major chromatogram peak) in the sample (Turle et al. 1989). Given that the congeners in environmental samples do not resemble commercial mixtures (Duinker et al. 1980), PCB analyses should be based upon the sum of a defined number of congeners in order to adequately predict the hazards associated with PCB pollution. Schwartz et al. (1987) analyzed PCB residues from fish and turtles using a PCA technique (SIMCA), and found that the sample residues were not adequately described by a single chromatogram peak or by an Aroclor mixture (like 1254/1260). Further, the authors indicated that such a representation of the PCBs found in the fish and turtle tissue would be both inappropriate and inaccurate. Duinker et al. (1991) identified six congeners (IUPAC numbers 28, 52, 101, 153, 138 and 180) whose sum is often used to represent "PCBs" in environmental samples. The authors suggested that the use of such a limited sum was confusing, and that any comparisons drawn between environmental compartments based upon such a sum were "principally wrong and misleading". The Duinker paper also reviewed the techniques which make individual congener analysis possible; and the authors recommended that such detailed identification be routinely performed on environmental samples in order to obtain information on "sources, transport mechanisms, sinks, accumulation and degradation", as well as to facilitate the development of predictive models.

It is important to characterize the exposure dynamics of individual PCBs as
toxicity varies among congeners, and measures of total PCB are not sensitive to these differences. In response to the recognized variation in congener partitioning between environmental compartments, as well as the poor representation of actual congener residues by Aroclor equivalents, I examined the distribution and exposure dynamics of 39 PCBs in the western basin of Lake Erie. I sampled species from different trophic levels in order to assess the relative importance of the two chemical exposure models (uptake from water and uptake from food) in the Lake Erie ecosystem. I also wanted to determine if total PCB concentrations increased with higher feeding levels, and if the relative concentrations and compositions of congeners differed among trophically diverse species. I performed my research in the western basin of Lake Erie as this lake sustains the largest freshwater commercial fishing industry in North America, producing in excess of 50 million pounds annually. The lake is also a favourite location for recreational sport fishing, thus human exposure to PCB pollution via Lake Erie biota is potentially high.
MATERIALS AND METHODS

Study Site

Middle Sister Island is located in the western basin of Lake Erie, approximately 13 km offshore of Colchester Ontario, (41°51'N 83°00'W), (Figure 2.1). It is a small island (2km by 1km), with Carolinian-type vegetation, and is inhabited by herring gulls, cormorants and ducks. All samples were collected on or near this site. Six separate sampling periods occurred between the months of May and October 1991. Middle Sister island was selected for this study because it is distant from any shoreline point sources. Additionally, a 'source-sink' relationship has been established between the Detroit River and Lake Erie (Carter and Hites 1991). The western basin is separated from the rest of Lake Erie by a chain of islands and an underwater sill, which act to prevent lake-wide mixing. Consequently, more than 77% of all Detroit River effluent remains within the western basin and Middle Sister Island is subject to continuous, low-level contamination.

Sampling Procedures

Piscivorous and planktivorous fish were collected for analysis using a 50m gill net with a 4.25 inch diameter mesh. This net was set overnight, and samples were collected at dawn the following day. Captured fish were placed on ice, and returned to the lab, where they were weighed, measured and dissected. Muscle tissue, liver and eggs (when present) were removed and frozen until analysis. Plankton were collected with a 125 um tow net, which was pulled behind the sampling vessel at 2-3 knots until
Figure 2.1  Sample collection site on Middle Sister Island in the western basin of Lake Erie.
a 5g sample was obtained. Sediment from 15m depths on the northern and southern shores of Middle Sister was sampled using a petite ponar, and was refrigerated until analysis. Unhatched gull eggs were collected from nests where other eggs were present, or from obviously abandoned nests. The eggs were wrapped in paper, and transported in an egg carton to prevent breakage. In the lab, eggs were wrapped in hexane-rinsed foil, labelled, and frozen until analysis.

Species collected

Herring gull eggs (*Larus aegentatus*) were collected from the Middle Sister Island colony (Plate 2.1). This species has been a biomonitor for the Great Lakes ecosystem since 1970 (Turle *et al.* 1989) and adult gulls and offspring were abundant on the island.

I sampled four different species of fish. Gizzard shad (*Dorosoma cepedianum*) are common in Lake Erie, but are not thought to be native. I captured large specimens of carp (*Cyprinus carpio*) from the southern shore of Middle Sister. This is an exotic species which was introduced to North America because of its suitability for pond culture and popularity as a food fish. Silver bass (*Morone chrysops*), more commonly known as white bass, were also sampled. This species spawns in early spring and swims onto shoals or into estuaries for spawning to occur. A large catch on May 23 was likely due to silver bass spawning in the shallow waters on the southern side of Middle Sister. Another popular game fish sampled was smallmouth bass (*Micropterus dolomieu*).
Plate 2.1  Herring gull nest and eggs
PCB Analysis

The liver and eggs of piscivorous fish species and the herring gull eggs were analyzed as wet weight samples of 2g. Fifteen grams of muscle from piscivorous fish species was initially extracted as three separate wet weight samples of 5g tissue per individual fish. After passage through anhydrous Na$_2$SO$_4$, these three samples were combined, to give one wet weight sample of 15g.

All samples were homogenized using a glass mortar and pestle. The glassware used was washed with soap and water, heated at 350°C overnight, and then rinsed three times with acetone, petroleum ether and, hexane (solvents used were pesticide grade).

Anhydrous sodium sulfate (previously heated at 650°C overnight) was added to tissue samples in a 1:4 by weight ratio (one part tissue to four parts Na$_2$SO$_4$). Samples were ground until homogeneous, and were then poured into a glass column (2cm x 35cm with a teflon stopcock). This column was plugged with dichloromethane-rinsed glass wool, and contained 10g sodium sulfate and 30mL DCM/hexane (50% v/v). Sample residue was removed from the mortar and pestle using 10g of Na$_2$SO$_4$, and three DCM/hexane rinses, which were all added to the top of the column. The sample was allowed to stand for one hour, and then was eluted at a flow rate of 1.5mL/min. In total, 310mL solvent was passed through the column, and was collected in a 500mL round bottom flask. 4mL isoctane (2,2,4-trimethylpentane) was added to the sample as a 'keeper', and it was then rotoevaporated to 5mL using a Buchler Rotoevaporator with a water bath temperature of 27°C. 2mL of sample was removed for lipid determination, and the remaining sample was further rotoevaporated to 2mL.
Sample cleanup involved Florisil (60-100 mesh) from Supelco, which was activated overnight at 130°C. This method gave high recovery efficiencies (greater than 90%) for all 42 congeneres (this total includes three coplanar PCBs which are discussed in Chapter Three), which were eluted in two separate fractions.

The lipid content was determined by letting 2 mL of tissue extract evaporate to dryness in a pre-weighed beaker. This was then dried at 105°C for one hour, placed in a desiccator for one hour and then re-weighed. The difference in the weight of the beaker represented the weight of lipid in that sample.

The 2mL rotovaporated sample was added to a glass column (1cm x 35cm with a teflon stopcock, plugged with DCM-rinsed glass wool), filled with 6g of florisil and approximately 2cm of Na₂SO₄ in hexane. The 500mL flask containing the sample was rinsed three times with hexane, and this was added to the column. In total, 50mL hexane was passed through the florisil in order to obtain the first fraction. This fraction was collected in a 250mL round bottom flask, and contained chlorobenzenes, pp'-DDE, trans-Nonachlor, photo-Mirex, Mirex, and PCBs (including mono-ortho substituted). Fraction 2 was collected in a 250mL round bottom flask, and was obtained by passing 50mL of 15% DCM/hexane (v/v) through the same column. The florisil was not allowed to dry out between fractions. The second fraction contained Oxychlordane, pp'-DDT, DDD, cis-nonachlor, and non-ortho substituted PCBs. Each fraction was individually evaporated (after the addition of 4mL isooctane as a keeper) to a final volume of 2mL which was then brought up to 5mL, 10mL, 25mL or 100mL with hexane. This volume varied with the lipid content of the sample tissue as high lipid levels interfere with the
sensitivity of the GC and ultimately ruin the column. Typically gull eggs and carp tissues were high in lipid and so were resuspended in larger volumes of hexane. Each fraction was analyzed separately on HP-5890 GC/ECD equipped with a HP-3396 integrator, and an HP-7673 autosampler.

Fraction 1 was compared with a calibrated standard solution (obtained from the CWS Laboratory, Ottawa), which contained TCB, QCB, HCB, OCS, trans-nonachlor, pp'-DDE, pp'-DDT, photomirex, mirex, and a mixture of Aroclors 1242:1254:1260 (1:1:1). The second fraction was compared with a standard mixture of 6 PCBs (#77, #126, #169, #189, #153, and #105), all at a concentration of 0.7ng/g.

Sediment samples were combined with Na\text{\textsubscript{2}}SO\text{\textsubscript{4}} in 1:4 parts, then layered in glass thimbles between pure Na\text{\textsubscript{2}}SO\text{\textsubscript{4}}. Each thimble was attached to a Soxhlet unit which had been pre-extracted with 150mL acetone/hexane (50% v/v), and samples were eluted with 300mL acetone/hexane (50% v/v) for 16 hours. The following day (when the soxhlet extraction was complete), the sediment sample was passed through a glass column (2cm x 35cm with a teflon stopcock, plugged with DCM-rinsed glass wool), which contained 40mL Na\text{\textsubscript{2}}SO\text{\textsubscript{4}}. The sample was eluted with 250mL hexane, and collected in a 500mL round bottom flask. The sample was rotoevaporated to 2mL, which was then passed through a similar column, filled with 24g florisil topped with 2cm Na\text{\textsubscript{2}}SO\text{\textsubscript{4}}. Fraction 1 was eluted using 200mL hexane, and Fraction 2 was eluted with 200mL DCM/hexane (15%v/v). Rotoevaporation followed. A blank of the same weight pure Na\text{\textsubscript{2}}SO\text{\textsubscript{4}} underwent the identical cleanup procedure once every six samples. Activated copper powder was added to each sediment sample when transferred to 10mL volumetric flasks,
in order bind sulphur. Sediment water content was determined by drying 5g sediment at 106°C for 48hr. Organic carbon content was then determined by placing the dried sample in the muffle furnace at 450°C overnight. Contaminants were quantified using GC-ECD techniques (see Lazar et al. 1992).

Statistical Analysis

All data were analysed using non-parametric statistical techniques. Often the variances were unequal, and distributions deviated from normal. Sample sizes within and among trophic levels were also consistently too small to use parametric tests effectively. This is one of the constraints when doing contaminant research, as the effort and cost per sample is large.
Results

Lake Erie biota and sediment samples contained detectable concentrations of PCBs (Appendix A). The data collected indicate that trophic enrichment of PCBs occurs in Lake Erie (Figures 2.2 and 2.4). The congener concentrations in the herring gull egg samples were two to three orders of magnitude greater than the concentrations in the sediment and phytoplankton samples, and at least one order of magnitude greater than the congener concentrations in the smallmouth bass muscle samples. There were significant differences in total PCB concentrations among phytoplankton, sediment, gizzard shad, carp, silver bass, smallmouth bass and gull egg samples (Kruskal Wallis H=19.07, 6DF, P=0.004). The phytoplankton samples exhibited the lowest total PCB concentrations (X±se=199.1±87.8 ug/kg organic carbon, n=3); while the herring gull eggs had the highest total PCB concentrations (X±se=239.4±74.0 mg/kg lipid, n=3).

‘Total PCB’ was determined by summing 39 ortho and three non-ortho substituted congeners for each compartment sampled. The fish species sampled displayed similar whole body concentrations of total PCB (Figure 2.4) with the exception of gizzard shad which had consistently lower total PCB concentrations. There were significant differences in total PCB concentrations among these different species (Figure 2.4, H=19.51, 6DF, P=0.004). No meaningful trophic patterns were discerned in the total PCB content for individual tissues (Figures 2.5-2.8).

The pattern of PCB congeners in samples varied with trophic level (Figure 2.3). Congeners with three chlorines represented a small percentage of total PCB in samples from all trophic levels. There was a significant difference in the percentage of
tetrachlorobiphenyls among the four trophic levels ($H=9.34, 3DF, P=0.025$). Tetrachlorobiphenyls comprised a greater proportion (42.1±6.8%) of the total PCB in the phytoplankton samples, than in gull egg (3.5±0.6%), sediment (18.8±1.5%), or bass muscle (12.7±1.4%) samples. The proportion of biphenyl molecules substituted with 7 and 8 chlorines was also found to be significantly different among the four trophic levels ($H=10.24, 3DF, P=0.025$; $H=10.24, 3DF, P=0.025$ respectively). Hexachlorinated congeners were not found in significantly different proportions among the four levels sampled ($H=6.54, 3DF, P=0.1$), although Figure 2.3 suggests that the hexa-substituted PCBs enrich more with increasing trophic level than the octa-biphenyls do. Certainly the hexa- and heptachlorinated biphenyls were enriched in gull eggs and each represented 37.5±1.0% of the samples' total PCB load. This percentage was higher than in the lower trophic samples, where hexachlorobiphenyls constituted 25.9±3.3%, 26.7±1.2% and 31.6±3.9% of the total PCB in phytoplankton, sediment and smallmouth bass muscle samples respectively. Heptachlorinated molecules represented 10.8±0.5%, 24.3±1.1% and 21.2±2.4% of the total PCB in phytoplankton, sediment and smallmouth bass muscle samples.

The pattern of PCB congeners found in the different species sampled was similar for the carp, silver bass, and smallmouth bass tissues (Figures 2.9-2.12). In gizzard shad samples, tetrachlorobiphenyls represented a much larger proportion of total PCB (22.5±0.9%, 21.6±2.1% and 21.8±3.4%, shad muscle, liver and egg respectively), than they did in the other fish samples (smallmouth and silver bass eggs had 17.2±1.4% and 16.6±0.7% tetrachlorobiphenyls but all other samples had less than 12.5% TeCB,
and gull eggs were as low as 3.4±0.6%). Within species, the differences in congener composition among tissues were not found to differ significantly (Friedman’s Test, F=7.40, 8DF, p=0.5; F=4.90, 8DF, p=0.9; F=3.77, 8DF, p=0.9; F=7.98, 8DF, p=0.5, for smallmouth bass, silver bass, carp and gizzard shad tissues respectively). Smallmouth bass muscle samples contained fewer hexachlorobiphenyls than did smallmouth bass egg or liver samples (31.6±3.9% compared with 37.9±1.7% and 37.6±1.5%). Additionally, smallmouth bass egg samples were lower in biphenyls with 8 chlorines (1.1±1.0% compared to 6.8±0.6% and 4.4±0.5%); and higher in tetrachlorinated molecules (17.2±1.4% compared to 12.6±0.5% and 12.7±1.4%), than were either smallmouth bass liver or muscle samples (Figure 2.9). Silver bass egg samples exhibited a higher proportion of tetrachlorobiphenyls than did silver bass liver or muscle samples (16.6±0.7 in contrast with 13.5±1% and 12.4±0.8%) (Figure 2.10). There were no differences in congener composition between carp muscle and liver samples (Figure 2.11). Gizzard shad liver samples had a smaller proportion of pentachlorinated molecules (20.1±0.4%), than did shad muscle or eggs (22.5±0.9% and 22.8±0.9%) (Figure 2.12).
Figure 2.2  Log$_{10}$ lipid-normalized mean contaminant concentrations for 39 PCB congeners in herring gull eggs, and smallmouth bass muscle, sediment and phytoplankton samples from the western basin of Lake Erie. Error bars represent ± 1 standard error.
Figure 2.3  Percent of tri-, tetra-, penta-, hexa-, hepta-, octa-, and nona-
chlorinated-biphenyls, in herring gull egg, smallmouth bass, sediment
and phytoplankton samples. Error bars represent ± 1 standard error.
Dominant congeners are listed to the right of the bar graphs.
Gull egg (n=3)
#180 > #153 > #138

Smallmouth bass muscle (n=3)
#138 > #153 and #180

Sediment (n=4)
#174 > #138 > #180

Phytoplankton (n=3)
#74 > #66/95 > #149
Figure 2.4 $\log_{10}$ lipid-normalized mean total PCB (sum of 42 congeners) for herring gull egg, smallmouth bass muscle, sediment and phytoplankton samples. Error bars represent $\pm$ 1 standard error.
Figure 2.5  $\log_{10}$ lipid-normalized mean contaminant concentrations for 39 PCB congeners in smallmouth liver, egg and muscle samples. Error bars represent $\pm$ 1 standard error.
Figure 2.6  \( \log_{10} \) lipid-normalized mean contaminant concentrations for 39 PCB congeners in silver bass liver, egg and muscle samples. Error bars represent ± 1 standard error.
Figure 2.7 $\log_{10}$ lipid-normalized mean contaminant concentrations for 39 PCB congeners in carp liver and muscle samples. Error bars represent ± 1 standard error.
Figure 2.8  $\log_{10}$ lipid-normalized mean contaminant concentrations for 39 PCB congeners in gizzard shad liver, egg and muscle samples. Error bars represent $\pm$ 1 standard error.
Figure 2.9  Percent of tri-, tetra-, penta-, hexa-, hepta-, octa- and nona-chlorinated-biphenyls, in smallmouth bass muscle, egg and liver samples. Error bars represent ± 1 standard error. Dominant congeners are listed to the right of the bar graphs.
Smallmouth Bass Muscle (n=3)
#105 > #138 > #153 and #180

Smallmouth Bass Eggs (n=3)
#138 > #153 > #149

Smallmouth Bass Liver (n=3)
#138 > #153 > #149
Figure 2.10  Percent of tri-, tetra-, penta-, hexa-, hepta-, octa- and nona-chlorinated-biphenyls, in silver bass muscle, egg and liver samples. Error bars represent ± standard error. Dominant congeners are listed to the right of the bar graphs.
Silver Bass Muscle (n=3)
#101 > #138 > #153

Silver Bass Eggs (n=2)
#138 > #153 > #149

Silver Bass Liver (n=3)
#138 > #153 > #182/187
Figure 2.11  Percent of tri-, tetra-, penta-, hexa-, hepta-, octa- and nona-chlorinated-biphenyls, in carp muscle and liver samples. Error bars represent ± 1 standard error. Dominant congeners are listed to the right of the bar graphs.
Carp Muscle (n=2)
#138 > #153 > #180

Carp Liver (n=2)
#153 > #138 > #149

PERCENT OF TOTAL PCB

NUMBER OF CHLORINES ON BIPHENYL RING
Figure 2.12  Percent of tri-, tetra-, penta-, hexa-, hepta-, octa- and nona-chlorinated-biphenyls, in gizzard shad muscle, egg and liver samples. Error bars represent ± 1 standard error. Dominant congeners are listed to the right of the bar graphs.
Gizzard Shad muscle (n=5)
#153 > #138 > #149

Gizzard Shad Eggs (n=3)
#138 > #149 > #153

Gizzard Shad Liver (n=3)
#138 > #153 > #180

NUMBER OF CHLORINES ON BIPHENYL RING
Discussion

Simple partitioning of contaminants between abiotic and biotic phases, should manifest in similar lipid-corrected and organic carbon-corrected chemical concentrations in all ecosystem compartments. In the aquatic food web of Lake Erie, individual PCB congeners exhibited different distribution patterns at the different trophic levels. Additionally, the concentration of total PCB (sum of 39 ortho and 3 non-ortho substituted congeners) varied among the study species. The animals sampled during this study exploited different food resources and occupied disparate habitats. The variability seen in PCB tissue residues can probably be attributed to species’ unique feeding behaviours and habitats, and to the physico-chemical properties of the congeners. The PCB dynamics of this food web are more complex than those predicted by thermodynamic models.

The food web sampled consisted of four fish species (gizzard shad, carp, silver bass and smallmouth bass), herring gull eggs, phytoplankton and sediment. Herring gull eggs represented the top trophic compartment in this system. Adult gulls are opportunistic feeders which consume a wide variety of food (including all fish sampled), and are considered good indicators of pollution (Gonzalez et al. 1991). The colony on Middle Sister Island was thought to reflect PCB contamination in the immediate area, because the fishing was abundant and gulls were observed successfully catching and feeding upon fish. Also, the colony was far enough from mainland garbage sources that I was confident the gulls were feeding mainly on fish. Gizzard shad are primary consumers which form a significant link in food webs that support recreationally and
economically important predators like bass and pickerel. Young shad feed on zooplankton, including protozoans, copepods and ostracods; however, as shad mature their gut lengthens, and their preferred food becomes algae. Adult shad are herbivorous, benthic feeders that are recognized to be one of the few species of freshwater fish that exist almost solely on vegetative material. Stomachs of gizzard shad may contain a lot of sediment; however, this is believed to be ingested accidentally. Carp also ingest large quantities of sediment as they suck up mouthfuls of detritus, expel it back into the water and then search the suspended material for possible food items. Their close association with the sediment make carp important links between the benthos and pelagic food webs. Carp are tolerant of, and seem to prefer waters rendered unsuitable by eutrophication. The species is considered to be detrimental to native fish and duck populations because it increases water turbidity, uproots plants and probably mobilizes sediment-bound contaminants. While usually not considered a palatable fish, carp do represent a significant source of protein for native Canadians and some fishing communities. Higher trophic levels in the Lake Erie food web were occupied by silver and smallmouth bass. Silver bass are common in clear waters and are thought to be visual feeders which are not attracted to their prey by scent. They tend to occupy the upper waters of lakes and are piscivorous. Juveniles feed on microscopic crustaceans and insect larvae, while adult silver bass mainly consume yellow perch and gizzard shad. Smallmouth bass are found in the shallow, rocky areas of lakes and rivers. They are ubiquitous predators which feed from the surface, in the water column and in the bottom substrate. Minnows and yellow perch dominate the diet of adult smallmouth bass; however, white sucker, silver
bass, gizzard shad and young smallmouth bass are also common prey.

The phytoplankton samples exhibited significantly lower levels of total PCB relative to the other samples, because of trophic dilution. As primary producers, the algae are exposed to hydrophobic contaminants through water alone; however, the levels of PCBs in water are often extremely low, and may be below the limit of detection. The lower concentrations of total PCB in gizzard shad samples in comparison to the samples from other fish species relates to the small amounts of contaminant in phytoplankton, which are the main food of shad.

Tetrachlorobiphenyls dominated the phytoplankton samples, because they have relatively low $K_{ow}$s, and follow thermodynamic partitioning, (Swackhammer and Skoglund 1993). The more highly chlorinated PCBs are less likely to bioconcentrate due to steric hindrance and sorption on particulate matter and dissolved organic matter. These higher $K_{ow}$ compounds may adsorb to particulates and plankton, then be ingested and biomagnified by fish. In contrast to the distribution of tetrachlorobiphenyls, hexa- and heptachlorobiphenyls were abundant in the gull egg samples because of biomagnification, as well as selective metabolism. Research suggests that PCBs which enrich exhibit chlorine substitutions at adjacent meta-para positions on at least one biphenyl ring (Borlakoglu et al. 1990; Boon et al. 1987; Safe 1985). As well, any congeners with 2,4,5 chlorine substitution on either ring are considered to be resistant to biodegradation (Gonzalez et al. 1991; Gagnon et al. 1990). PCB congeners which showed the greatest enrichment in the gull egg samples, were $180 > 153 > 138$. These congeners are major components of Aroclors 1254 and 1260, and are chlorinated
at adjacent meta-para carbon atoms. Lower chlorinated isomers (such as tetra- and pentachlorobiphenyls), will often have adjacent carbon atoms without chlorine substitutions, and so will be metabolized. This expectation seems to have been fulfilled with congener #44 in the gull egg samples, as it is not detectable. PCB #44 is abundant in the tissues of all the fish species, but has 2,2',3,3' chlorine substitution, and so appears to be metabolized by the gull. This is not to suggest that enrichment of PCBs in biota only occurs in the absence of metabolism; rather, these substitution patterns are more resistant to biodegradation and thus persist in tissues at higher levels than do congeners with vicinal hydrogens or fewer chlorine substitutions.

Annual CWS monitoring of PCBs in herring gull eggs documented similar congener patterns (low proportions of tetra-, higher percentages of hexa-and heptachlorobiphenyls), as well as a shift in dominance from #138 > #153 > #118 > #180 during the early 1970s, to #153 > #180 > #138 in the late 1980s. The decline in PCB #118 is significant, as this congener lacks 2,4,5 chlorine substitution on one ring, and is thought to be metabolized by a different group of enzymes. The total PCB concentrations observed during CWS monitoring (Turle et al. 1989) were similar to the values I recorded which suggests that the PCB levels in Lake Erie have not changed appreciably in the last decade. An alternative explanation is that the dynamics of PCBs in Lake Erie have shifted since the invasion of the zebra mussel. This species may have altered the plankton and particulate matter concentrations in the water column, such that more PCBs became bioavailable, and are now manifested as increased PCB levels in predatory animals.
The composition of 'total PCB' was different for each trophic level, and cannot be considered a consistent measure. Additionally, where #138 is used to estimate Aroclor 1254:1260 mixtures ('total PCB' before individual congener analyses were possible), the estimate is twice as large as that obtained by summing individual PCBs. Using the former CWS methodology, my mean concentration of 'total PCB' in gull eggs based upon Aroclor equivalents, is 512.28 mg/kg lipid (compared with 239 mg/kg obtained through the quantification and sum of 42 individual PCBs). Additionally, given that #138 is a dominant congener in all samples, it reveals few differences in PCB patterns among species. The use of one congener to estimate total PCB load in tissues provides little useful information concerning differential bioaccumulation and selective metabolism, and so is not ideal for hazard assessment and prediction of long term trends.

Figures 2.2 and 2.5-2.8 display the concentrations of 39 congeners (the non-ortho PCBs are not shown) in the compartments sampled. These graphs reveal some interesting anomalies, which suggest just how complex contaminant partitioning is. Gizzard shad are primary consumers which graze 'sedimented' algae; however, the shad tissues sampled contained detectable levels of several congeners not present in the phytoplankton samples. PCB #31 has a log $K_{ow}$ of 5.62, and so may be bioconcentrated by shad. PCBs #185, #200, #195, and #206 were not detected in phytoplankton, but were present in all gizzard shad tissues. It is unlikely that water is a significant exposure route for these PCBs, as they all have $K_{ow}$s greater than 7.1 and are either hepta- or octachlorobiphenyls. These congeners are extremely hydrophobic, and their large molecular size likely impedes their transport across gill membranes. PCB #118 is also
absent in algae but present in gizzard shad. However, it is different from the other four congeners, because it has a lower $K_{ow}$ (6.7) and has a planar configuration, which enhances membrane adsorption and uptake (Shaw and Connell 1984). Gizzard shad may accumulate these PCBs from ingested sediment; however, congeners #185, #200 and #195 were not present in the sediment samples analyzed. Anomalies like these reveal why predictions of activity (e.g. Williams et al. 1992) and QSARs are limited approaches to contaminant dynamics, and reinforce the need for congener-specific analyses.

Interestingly, species' tissues exhibited similar concentrations and distributions of congeners. Fish may eliminate less hydrophobic contaminants via their gills, however they are not efficient at metabolizing PCBs (Hutzinger et al. 1972). Equilibrium partitioning among tissues is thought to be an important route to reducing potentially toxic levels of contaminants. The differences that were observed in species' tissue concentrations could have resulted from depuration into the egg masses, elevated liver enzyme activities, variable lipid levels, or chlorine content. Some studies suggest that lipids do play a significant role in the redistribution of PCBs. However, Oliver and Niimi (1983) documented differences in whole body and muscle PCB concentrations which were independent of lipid content. In contrast, Gagnon et al. (1990) demonstrated that up to 91% of the variation in total PCB concentrations for zooplankton and two species of fish could be explained by variation in the lipid content of the organisms.

The PCB concentration in tissues from all fish species were well above the limit of 0.1 mg/kg wet weight set by the International Joint Commission for the protection of predators (International Joint Commission 1978). Some species' concentrations were also
considerably higher than the U.S. Food and Drug Administration guideline of 2 mg/kg PCBs in fish sold commercially (Cunningham et al. 1990). These levels of contamination are of concern because of the associated biological effects. However, the PCB residues are also troublesome, because they vary among environmental compartments, and do not resemble the original sources of PCB pollution. Congeners vary in toxicity, and if those PCBs which are resistant to biodegradation are also the most hazardous to humans and wildlife, it is important to characterize the distribution, abundance and mechanism of biological action of selectively enriched congeners.

In summary, Lake Erie biota bioaccumulate high levels of PCBs. The congeners which animals are exposed to differ from those retained in their tissues. Trophic enrichment, metabolism, and selective uptake are all processes which alter the PCB pattern in different species, and make the analysis of individual PCB congeners in samples a necessary component of biomonitoring.
CHAPTER THREE

The Distribution and Exposure Dynamics of Three Coplanar PCBs in Lake Erie Sediment and Biota

Introduction

The need for congener-specific analysis is reinforced by evidence which indicates that PCB toxicity is related to structure, and is correlated to coplanar configuration (Tanabe et al. 1987). Several congeners exhibit this coplanarity due to non-ortho substitution in the biphenyl rings (Figure 3.1). The toxicity of these PCBs is augmented by chlorine substitution at both meta and para positions. Three congeners which display this substitution pattern and have been found in the environment are #77, #126 and #169. The toxic behaviour of these coplanar PCBs resembles that of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). More commonly known as dioxin, TCDD has been associated with wasting syndrome, thymic atrophy, reproductive toxicity and teratogenicity in various species (Hoffman et al. 1987, Harris et al. 1985 and Safe 1984).

TCDD has been shown to maximally induce activity in one member of the multienzyme mixed-function oxygenase (MFO) system. MFOs are important in the synthesis and metabolism of steroids, fatty acids and prostaglandins. However, it is their role in converting xenobiotics into more water-soluble, excreted metabolites that makes them significant to ecotoxicology (Rattner et al. 1989). Occasionally MFOs produce metabolites which are more reactive than the parent compound, and it is these products which lead to many of the syndromes mentioned.

MFO induction occurs through the reversible binding of a TCDD-like compound
Figure 3.1  Three coplanar PCBs, #77, #126 and #169, and 2,3,7,8-tetrachlorodibenzo-p-dioxin.
2,3,7,8-TCDD
to a cytosolic receptor, followed by translocation of the receptor-molecule complex into the nucleus. The complex then acts to stimulate increased metabolism of xenobiotics. Structure-activity analyses reveal that the affinity with which compounds bind the receptor is dependent upon planarity. Specifically, active MFO inducers must be substituted at both para, at least two meta, and less than two ortho carbons (Smith et al. 1990, Safe et al. 1985). Receptor binding affinity is strongly correlated with the level of enzyme induction and subsequent toxicity (Safe et al. 1985). Coplanar PCBs induce MFOs, and are thought to represent a greater hazard to humans and wildlife than TCDD, because they are present in the environment at 1000 fold higher concentrations.

It is possible to express the toxicity of coplanar PCBs in terms of dioxin-like activity. Toxic-equivalency-factors (TEFs) are the product of a compound's environmental concentration, and it's ability to induce MFOs (Tanabe et al 1989). TEFs have been determined for many TCDD-like xenobiotics. The non-ortho congener #126 is considered to be the most toxic PCB, and has 40% of the binding affinity (hence induction and toxicity) of dioxin, whereas #77 and #169 have only 0.3% and 0.2% of TCDD binding affinity respectively (Smith et al. 1990, Safe 1987). It can be appreciated that if PCB #126 is present in the environment at ten times the level of TCDD, then it will represent four times the toxicity of dioxin based on TEFs.

Despite representing only a small proportion of commercial mixtures (less than 1% w/w of Aroclors and Kaneclors; Kannan et al. 1987), coplanar PCBs are ubiquitous in the environment, and appear to enrich in biota relative to those congeners that dominate Aroclors and Kaneclors (Smith et al. 1990, Kubiak et al. 1989 and Tanabe et
al. 1987). This may be due to species’ inability to metabolize coplanar PCBs. The presence of adjacent chlorinated ortho and meta carbon atoms on at least one ring of the biphenyl molecule are essential for metabolism (Tanabe et al. 1989).

Studies on mussels documented significantly longer biological half-lives, clearance rates and time to uptake equilibrium for coplanar PCBs (Kannan et al. 1989). It appears that receptor interaction may alter the kinetics and partitioning of PCBs #77, #126 and #169. The method originally developed for extraction of coplanar PCBs was based on the high affinity of carbon for planar aromatic compounds (Tanabe et al. 1987b). Animal lipid is high in carbon, and the strong attraction of coplanar for this phase may prevent animals from mobilizing and eliminating coplanar PCBs.

The literature which documents coplanar PCBs in wildlife does not address the relative distribution of these congeners in food chains. We assessed the relative abundance of IUPAC #77, #126, and #169, along with 39 other PCB congeners in various species from Lake Erie. If aquatic organisms are exposed to PCB primarily through water, congeners 77, 126 and 169 should partition according to their respective $K_{ow}$s, where $L_f \times BCF = K_{ow}$, and $L_f \times BCF$ is the lipid corrected bioconcentration factor. If $K_{ow}$ is not the most important factor controlling PCB partitioning, individual congeners with similar hydrophobicity may experience enhanced accumulation through stereochemistry, and therefore become trophically ‘enriched’.
Materials and Methods

Study Site and Sampling Procedures

Middle Sister Island in the western basin of Lake Erie was the site for collection of unhatched herring gull (*Larus argentatus*) eggs, sediment and phytoplankton (Figure 2.1). Piscivorous and planktivorous fish species were also collected for analysis as described in the methods section of Chapter Two. These species included carp (*Cyprinus carpio*), silver bass (*Morone chrysops*), smallmouth bass (*Micropterus dolomieui*), and gizzard shad (*Dorosoma cepedianum*).

PCB Analysis

All of the samples collected and used for the determination of total PCB concentrations and congener-specific distributions were also used to quantify the residues of three coplanar PCBs. The exposure dynamics of congeners #77, #126 and #169 were also examined using these samples, and were compared with that of PCB #138.

The cleanup and extraction of samples was identical to the procedure outlined in the methods section of Chapter Two. Fraction two (using 15% DCM/hexane as the elution solvent) yielded the non-*ortho* substituted PCBs. The activated florisil approach was our preferred coplanar extraction technique, because it gave high recovery efficiencies, and results did not differ significantly from those obtained using carbon columns (Lazar et al 1992). Additionally, the florisil approach involved fewer steps and used less hazardous solvents. Co-elution of congener #77 and congener #110 was resolved by the Florisil technique, and did not present a problem (Lazar et al 1992;...
Storr-Hansen et al. (1992). Following analysis on a HP-5890 GC/ECD equipped with a HP-3396 integrator, and an HP-7673 autosampler, Fraction two was compared with a standard mixture of 6 PCBs (#77, #126, #169, #189, #153, and #105), all at a concentration of 0.7 ng/mL.
Results

Lake Erie biota, and sediment samples contained detectable concentrations of PCBs (Appendix A). The relative concentrations of the three coplanar PCBs in the lipid of all species sampled were usually in the order of \#77 > \#126 > \#169. However, in herring gull eggs, the concentrations of \#126 and \#169 were similar (Figure 3.2). PCB \#138, which was selected as a representative di-ortho substituted congener based upon Principal Components Analysis (Table 3.1), was found in all samples at levels one to two orders of magnitude greater than those of the non-ortho PCBs. The variability of the contaminant residue data was great. Sample distributions deviated from normal and sample variances were unequal (using Fmax-test and Bartlett’s test) despite log-transformations. Data were therefore analysed for differences in coplanar concentrations among species using the Kruskal-Wallis test, which is a nonparametric equivalent of a one-way ANOVA. Data were also assessed for differences in the levels of coplanars between tissues and species, using Friedman’s method, which is comparable to a two-way ANOVA. Non-transformed means and standard errors are recorded in Appendix A.

Concentrations of PCB \#77 (ug/kg lipid) were not significantly different among samples (H₃=11.87, P=0.065). There were significant differences in the concentrations of PCB \#126 among the six samples (H₃=17.64, P=0.001). No a posteriori tests were performed to sort out which samples were significantly higher; however, as Figure 3.2 indicates, the herring gull eggs had elevated levels of PCB \#126 relative to the sediment and the other species sampled (for gull eggs, sediment, silver bass and smallmouth bass respectively \(X\pm se = 114.0\pm 58.4, 4.8\pm 3.0\) ug/kg, and below the limit of detection
[0.02ug/kg] for both fish species). There were also significant differences among the six samples for PCB #169 (H₃ = 14.54, P = 0.025) but once again no a posteriori tests were carried out to pinpoint the specific differences. It is apparent from Figure 3.2 that congener #169 was only detected at high levels in the herring gull egg and sediment samples. The mean concentrations for the gull eggs and the sediment were 21.9±0.3 ug/kg and 19.4±1.3 ug/kg respectively, compared to the next highest value of 1.43±1.4 ug/kg seen in the silver bass muscle samples. Concentrations of PCB #138 were found to be significantly different among phytoplankton, sediment, the four fish species and the herring gull eggs (P = 0.003, H = 19.94, 6DF). The herring gull eggs and carp muscle samples exhibited much higher mean concentrations of #138 than did the other samples (8927.0±6557.2 and 2348.0±76.0 ug/kg compared with 1230.7±194.9, 412.7±88.0, 343.8±143.0, 57.6±6.3 and 13.6±4.2 ug/kg for smallmouth bass muscle, silver bass muscle, gizzard shad muscle, sediment and phytoplankton samples respectively. Phytoplankton samples yielded such low levels of coplanar PCBs, it was impossible to quantify them adequately. The contaminant relationships in Figure 3.2 are limited by the absence of forage fish data. These species were never collected at Middle Sister Island, despite extensive seining.

Coplanar PCBs, as well as ortho-substituted PCBs, were present in fish liver and eggs (Figure 3.3). No eggs were obtained from captured carp. A Friedman two-way analysis of variance indicated highly significant differences between levels of congener #77 in the livers and eggs of shad, silver bass and smallmouth bass (F = 17.63, 1DF, P < 0.001). Concentrations of PCB #138 were also significantly different with
respect to levels in eggs and livers from the three fish species (F=18.72, 1DF, P<0.001). Congener #169 exhibited marginally significant differences in egg and liver residue levels in the three species (F=4.63, 1DF, P=0.043), while #126 was not found to be significantly different between tissues among species (F=3.45, 1DF, P=0.07).

Data from egg and muscle tissues were compared for gizzard shad, silver bass, and smallmouth bass, in order to assess the contaminant load being passed on to the young-of-the-year (Figures 3.2 and 3.3). There were no significant differences found between residues of congener #169 and #126 in eggs and muscle (Friedman two-way analysis of variance, #169: F=1.71, 1DF, P=0.21; #126: F=3.7, 1DF, P=0.15). PCBs #77 and #138 exhibited significantly different concentrations in eggs and muscle tissue for shad, silver bass and smallmouth bass (#77: F=11.14, 1DF, P<0.001; #138: F=14.0, 1DF, P<0.001). Smallmouth bass eggs were more contaminated with both #77 and #138 than smallmouth bass muscle, while #77 concentrations were elevated in silver bass eggs when compared with silver bass muscle. The opposite trend was seen for congener #138 in silver bass tissue. Gizzard shad muscle was slightly more contaminated with #77 than shad eggs; however, the eggs from shad had considerably higher levels of #138 than did shad muscle.

The percent of coplanar congeners in gull egg, fish muscle, phy o plankton and sediment samples is illustrated in Figure 3.4. The proportion of total PCB congeners that are coplanar, decreases with increasing trophic level. The summed concentrations of #77, #126 and #169 were significantly higher in sediment than in biota (Mann Whitney
U < 0.001, P=0.05), and slightly higher in silver bass and smallmouth bass than in gull eggs (H=6.60, 2DF, P=0.08). The smaller proportion of coplanar congeners in gull eggs still represents a hazardous level of toxic equivalents. In other words the gull eggs are so contaminated with PCBs that the coplanar congeners represent a small proportion of total PCB. This apparently small percentage actually represents a mean sum of coplanar congeners in gull egg samples equal to 220.0±76.0 ug/kg. If this amount were comprised of 50% congener #126 (i.e. 110ug/kg) and we recognize that #126 binds the Ah receptor with 40% of the affinity exhibited by 2,3,7,8-TCDD, then per gull egg there is the equivalent of 44 ug/kg 2,3,7,8-TCDD in terms of Ah receptor-mediated biological activity. This is certainly a level of contamination of concern.
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* PCB 31 frequently co-elutes with PCB 28, thus the assignment to a separate component.
Figure 3.2 Log_{10} lipid-normalized PCB concentrations in Lake Erie phytoplankton (PHYTO), sediment (SED), carp, gizzard shad (GS), silver bass (SB), smallmouth bass (SMMB), and herring gull eggs (EGG). The values illustrated represent means ± 1 standard error.
Log concentration (ng/g lipid)

PCB#77

PCB#126

PCB#169

PCB#38

Sample
Figure 3.3 \( \log_{10} \) lipid-normalized PCB concentrations in liver and egg tissue of carp, gizzard shad (GS), silver bass (SB), and smallmouth bass (SMMB), from Lake Erie. Means ± 1 standard error are shown.
Log concentration (ng/g lipid)
Figure 3.4  The percent of coplanar congeners in sediment (SED), phytoplankton (PHYTO), gizzard shad (GS), carp, silver bass (SB), smallmouth bass (SMMB), and gull egg (EGG) samples. Mean percents ± 1 standard error are shown.
Discussion

Principal Components Analysis of the congener distribution in Lake Erie sediment and biota revealed some interesting, but not unexpected associations. Factor 1 included only those PCBs with some degree of ortho chlorine substitution. Log K_{ow}s were lower for congeners grouped in factor 1. Congeners in this group all have log K_{ow}s equal to or below 7.11; while factor 2 congeners have log K_{ow}s greater than 7.2 and are highly chlorinated. These two factors reinforce that PCB partitioning is strongly influenced by hydrophobicity and by the amount and the location of chlorine substitution. PCBs #169 and #189 (which grouped under factor 3) exhibit identical patterns of substitution, except that #189 has a chlorine at one ortho carbon. They are enriched with chlorines at meta-para positions, and have log K_{ow}s of 7.42 and 7.71 respectively. #77 and #60 also display similar patterns of chlorine substitution, except that #60 is a mono-ortho biphenyl. PCB #126 appears to partition separately from all other congeners, which may relate to its high-affinity interaction with biological receptors. In summary, PCA indicates that the non-ortho PCBs have a different distribution compared with all other congeners, and that their activity in environmental phases cannot be correlated with that of ortho-substituted congeners, including PCB #138.

It was previously thought that organochlorines with log K_{ow} < 6 were subject to few inter and intraspecific differences, as they were relatively water soluble (Hebert and Haffner 1991). It appears that there may, in fact, be a narrow cutoff between 'water soluble' and sorbed. Congener #77 has a log K_{ow} of 5.6 - 6.7 (Hawker and Connell 1987; Shiu and Mackay 1986) and is ubiquitous among environmental samples, whereas,
PCB #126, with a log K_{ow} of 6.89 is only found in sediment-associated species (like gizzard shad and carp), and appears to be further enriched in top predators (herring gull eggs). Congener #126 is not available for uptake from water, as levels were non-detectable in the pelagic silver bass and smallmouth bass. Conversely, levels of #126 were elevated in carp, which are detritivores, and in gizzard shad, which are planktivorous benthic feeders that also consume large quantities of sediment while feeding (Scott and Crossman 1975).

It is known that sediment acts as a sink for hydrophobic contaminants, and that this role is emphasized when chemicals are highly sorbed (Swackhammer and Armstrong 1988). In North America, as regulations become more stringent and PCB input decreases, the sediment will become an increasingly important PCB source. Food web transfer among benthic and pelagic species represents a significant process in PCB pollution dynamics. Congener #169, with a K_{ow} of 7.42, exhibits a similar distribution to #126, and was found at high levels in sediment and herring gull eggs. Interestingly, PCB #138, which has a log K_{ow} of 6.62-7.44, was found in all samples, and was present at similar levels in the fish species sampled despite differences in habitat and resource use among these species. Based upon K_{ow}, congeners #126 and #138 should exhibit similar exposure dynamics. These data reiterate the problems inherent in assessing PCB contamination with a discrete mixture of congeners acting as surrogates for 'total PCB'.

There is a possibility that the toxicokinetics and accumulation of coplanar PCBs in Lake Erie biota could be influenced by the relative affinity with which these compounds bind to cytosolic MFO receptors. Similar congener distributions to those
observed for fish muscle occurred in the egg and liver tissue (i.e. congeners #138 and #77 more abundant and more consistently present in all tissue samples than #126 and #169). The higher concentration of congeners in eggs may also result from depuration. Transplacental and lactational transfer have been documented in mammals (Tanabe et al. 1987). However, elevated lipid levels in fish eggs may cause mobilization of PCBs as equifugacity is approached.

Williams et al (1992), argued that congener-specific analyses are not necessary due to 'nearly accurate' estimations based upon correlating burdens of coplanar PCBs with the concentration of total PCBs. My research revealed, however, that the percent of total PCB contributed by coplanars decreases with trophic level (despite the fact that the gull eggs had the highest lipid-normalized concentrations of coplanar PCBs). In other words, total PCB cannot be used to predict the concentration of coplanars in a sample, because the relationship shifts with different species. Tanabe et al. (1987) also noted strong correlations between the concentration of total PCB and the concentration of coplanar PCBs in samples. However, the authors did not suggest abandoning isomer-specific determination. Rather, they strongly recommended individual congener monitoring, because the composition of coplanars differed significantly among five species of marine mammals, three species of terrestrial mammals, and fish.

There were few differences in the distribution of ortho-substituted PCBs between tissues in fish (see Chapter Two). However, the coplanar PCBs did exhibit variable partitioning between muscle, liver and egg tissues. It has been shown that hepatic sequestering of TCDD occurs in rats (due to the high number of Ah receptors in the liver
relative to other tissues) and is manifest in decreased dioxin distribution to extrahepatic tissues (Birnbaum 1993). While livers were not always more heavily burdened with coplanar congeners, this research suggests that the differential partitioning seen in the non-ortho PCBs, when compared with total PCB, may reflect Ah receptor interaction effects.

Coplanar PCBs are present in Lake Erie biota at significantly elevated levels. Individual PCB congeners do not partition as a group within the aquatic environment. Many PCB congeners that were quantified exhibited significant trophic enrichment. The literature suggests that a combination of high exposure concentrations and extended duration of exposure result in slower PCB depuration rates and elevated body burdens. The levels of PCBs quantified in sediment, fish and birds suggest that this pattern is occurring in Lake Erie, and thus contamination of wildlife is not likely to decline soon. This study confirms ecosystem contamination in the western basin of Lake Erie by the hazardous coplanar PCBs. My research also emphasizes the need for individual congener research, for without a comprehensive data base on PCB congener partitioning and toxicity it is impossible to establish cause and effect relationships between residue levels and observed pathologies.
CHAPTER FOUR

The Relative AHH Induction Potential of Three Coplanar PCBs in Mouse, Rat and Human Cell Lines

INTRODUCTION

Aryl Hydrocarbon Hydroxylase (AHH) is a metabolic enzyme, which belongs to the family of cytochrome P450-mediated biotransforming enzymes, collectively known as 'mixed function oxygenases' (MFOs). These membrane-bound monooxygenases are responsible for the metabolism of a wide variety of substrates, including therapeutic drugs, environmental contaminants, and naturally occurring cellular compounds (Giclen and Nebert 1972). The monooxygenases are 'inducible' or adaptable enzymes which increase in activity in response to exposure to inducer substances. This induction was initially discovered through the altered pharmacologic responses of laboratory rats, and through the changed efficacy of certain drugs in human patients (Remmer 1962, in Okey 1990). Today, MFO induction, and specifically increased AHH activity, have new ecotoxicological significance, as elevated levels of AHH indicate prior or chronic exposure to environmental pollutants. Cigarette smokers and Great Lakes fish are two common examples of organisms with increased AHH activity in response to repeated exposure to polyaromatic hydrocarbons (PAHs) and organochlorines (OCs) (Luxon et al. 1987).

The mechanism of AHH induction has been studied using cell culture. The presence of cytosolic and nuclear Ah receptors, the translocation of the receptor from
cytosol to nucleus, and the physical characteristics of the receptor were elucidated by Okey et al. (1980) using Hepa-1 (mouse hepatoma) and H4IIIE (rat hepatoma) cell lines. The H4IIIE line was considered to be a more relevant system (medically) following this publication, and became the standard cell line for AHH study. Hepa-1 cells were highly inducible, and unlike H4IIIE, exhibited both nuclear and cytosolic receptors at 37°C; however, the murine strain was not considered to be representative of the human system. Subsequently, AHH induction was discovered in two human hepatoma cell lines, HepG2 and Hep3B (Labrizzo et al. 1989). A binding component similar in structure and function to the mouse Ah receptor was also demonstrated. HepG2 and Hep3B now represent relevant human model cell systems which facilitate the study of the regulation of AHH induction, as well as the potentially toxic and carcinogenic effects of environmental pollutants on humans.

MFOs may be identified by their 'inducer category'. These categories are named for compounds which maximally induce specific enzyme activity. Polychlorinated biphenyls are known to induce and be biotransformed by two of these categories. Ortho-substituted biphenyls are considered to be 'phenobarbitol-like', and induce P450IIB1 and IIB2 (Safe et al. 1985), while coplanar PCBs belong to the class of methylcholanthrene-like compounds, and induce P450IA1 and IA2. AHH is coded for by the P450IA1 gene, and so tends to increase in response to planar polycyclic aromatic hydrocarbon exposure (Nebert et al. 1987).

The proposed mechanism of P450IA1 (AHH) induction involves the binding of an inducer to the cytosolic Ah receptor, and subsequent translocation of the inducer-
Figure 4.1  The proposed mechanism by which methylcholanthrene-like compounds induce P450IA1.
receptor complex into the nucleus (Figure 4.1). Inside the nucleus the inducer-receptor complex interacts with at least three and possibly more 'xenobiotic responsive elements' (XREs) located upstream of the cytochrome P450IA1 gene. Association of this complex with these specific DNA sequences leads to the activation of structural and regulatory genes, increased transcription, translation, and ultimately elevated monooxygenase catalytic activity. The receptor is thought to be the major regulatory gene product, and intracellular receptor concentrations also increase in response to induction (Nebert and Gonzalez 1987). The overall structure and function of the Ah receptor is similar to that of steroid hormone receptors, but no steroids are known to compete for AhR binding. Endogenously produced substances which do bind the Ah receptor include indoles, thyroxine, fatty acids and photooxidation products from histidine and tryptophan (Rannug et al 1987; Paine and Francis 1980).

Ligands and substrates occupy the same Ah receptor binding site (Ullrich 1979), thus inducing compounds frequently stimulate their own metabolism. One inducer, which is not metabolized or transformed by AHH (except in beluga whales), is 2,3,7,8-tetrachlorodibenzo-p-dioxin (Barnes 1991). TCDD, a by-product of the synthesis 2,4,5-trichlorophenoxyacetic acid ('agent orange'), is the most potent P450IA1 inducer known, and binds the Ah receptor with 30,000 times the affinity displayed by the prototype inducer, methycholangrene (Poland and Glover 1974). It is generally accepted that AHH-related toxicity is mediated by the Ah receptor, and that those compounds which bind to the receptor with high affinity produce the most induction, and are the most hazardous to the cell. Conney (1982)
described the most potent P450IA1 inducers as coplanar, hydrophobic molecules with halogen substitutions. The coplanar PCBs IUPAC #77, #126 and #169, closely match the ligand description, and toxic equivalence research indicates congener #126 binds the Ah receptor with 40% of the efficacy displayed by TCDD (Safe 1990). Additionally, research suggests that PCBs are important inducing agents only when present at high concentrations relative to dioxin (Bellward et al. 1990). My data, as well as the literature (Schwartz et al. 1988, Kubiak et al. 1989), confirms the abundance of coplanar PCBs in the general environment. Given the low levels of TCDD in wildlife (responsible for less than 10% of the measured AHH activity in Forsters Terns, Kubiak et al. 1989), it appears that the coplanar PCBs do play an important role in P450IA1 induction.

Mixed function oxygenases catalyze biological oxidations of endogenous and foreign compounds through the direct incorporation of elemental oxygen into the substrate molecule. The products associated with MFO activity are innocuous and are more water soluble than the parent compound. MFOs alter substrates so that they are suitable for phase II conjugation reactions and are more easily eliminated (Rattner et al. 1989). However, the enzyme pathway which solubilizes foreign compounds produces highly reactive intermediates which are mutagenic and/or carcinogenic to the cell (Ioannides and Parke 1987).

The pathway by which AHH biotransforms xenobiotics has been widely studied using rat liver microsomes and Benzo[a]pyrene (B[a]P) as substrate (Phillips 1983). B[a]P is a common environmental carcinogen that has been associated with a diverse array of combustion products. The strong fluorescence of B[a]P's phenolic metabolites
has made it the basis for the AHH assay (Yang et al. 1978). The elucidation of B[a]P AHH-mediated biotransformation reveals how a highly conserved metabolic pathway produces harmful metabolites. The initial step in B[a]P breakdown is AHH-catalyzed oxidation to 7,8-epoxide (Figure 4.2). The epoxide may rearrange nonenzymatically to form innocuous, excretable phenols; or, it may be combined with water-soluble conjugates via glutathione S epoxide transferase (GST), and be excreted. Alternatively, the epoxide may be (stereospecifically) converted by epoxide hydrolase (EH) to a trans-7,8-diol. The diol is further oxygenated, predominantly at the 9,10 double bond, to form a diol epoxide. Vicinal diol epoxides are the most chemically reactive epoxide species formed. Oxidation forms two enantiomers, the trans-7,8-dihydroxy-anti-9,10-epoxy tetrahydrodiol, and the -syn- isomer. It appears that mutagenicity is stereoselective, as the anti-diol epoxide is the predominant form found to bind, in vivo, the nucleic acid of mammalian cells (Yang et al. 1978). The diol epoxides may each be hydrolyzed in aqueous media to a pair of tetrols, which then are reduced by NADPH or NADH to triols, and excreted.

The oxidative metabolism of polychlorinated biphenyls, appears to depend upon the presence of adjacent meta-para hydrogens (Borlakoglu et al. 1990). This configuration facilitates the AHH-catalyzed insertion of an atom of oxygen, and subsequent formation of an epoxide. Additionally, the oxidation of PCB congeners is favoured at meta-para carbons in the least chlorinated phenyl ring. Increasing chlorination of congeners, reduces the probability of monooxygenase metabolism, due to steric hindrance.
Figure 4.2  The biotransformation of Benzo[a]pyrene by Mixed Function Oxygenases (MFO), leading to the formation of reactive and potentially damaging intermediate compounds, as well as excretable tetrols and triols.
The relative proportion of innocuous and reactive metabolites formed during MFO activity, is closely linked to the P450 species induced, and is dependent upon the respective levels of conjugating enzymes (EH and GST) and monooxygenases. Thus, induction or inhibition of certain enzymes alters the balance of the metabolic pathway, such that pretreatment of laboratory rats with 3-methylcholanthrene (MC) led to a significant increase in the formation of the reactive anti-diol epoxide relative to levels in untreated rats. It has been suggested that, although EH, GST, and UDP-glucuronyltransferase are induced by MC-type compounds (Centley and Oesch 1982; Owens 1977), the degree of inducibility of P450s is often higher and this results in a significant imbalance between the rate of formation of reactive metabolites, and the inactivation of these products.

The genotype of the exposed animal and the toxicant exposure route also effect the outcome of P450 induction. Research on laboratory mice indicates that the inductive phenotype is an autosomal dominant trait, and Okey et al. (1979) have identified responsive and nonresponsive mice. In responsive animals, if the precarcinogen is placed in direct contact with tumor-susceptible tissue (skin), elevated AHH activity at this site leads to the formation of ultimate carcinogens. Conversely, if MC-type inducers are consumed, or injected intraperitoneally, the responsive animal experiences a decreased incidence of cellular damage, due to the 'first pass effect'. Also known as presystemic drug elimination, this situation involves highly induced hepatic AHH, which efficiently metabolizes the PAH/OC before it reaches a site of action.

The induction of AHH has been widely researched due to its role as an interface
between environmental contaminants and the cell. It is considered an extremely sensitive biomarker of contaminant exposure and environmental quality, and has become a popular measure of exposure in wildlife (Monosson and Stegeman 1991; Ankley et al. 1991; and Masfaraud et al. 1990).

The AHH assay is a unique and sensitive method of monitoring the effects of pollutants upon individuals before ecosystem-wide effects occur. The assay is performed on cells grown in easily controlled microenvironments. This facilitates the study of dose-response relationships for single contaminants or complex mixtures, and may contribute to an understanding of chemical interactions. In response to the hypothesized role of the coplanar PCBs as significant environmentally-occurring AHH inducers, I set out to quantify dose-response relationships for individual PCB congeners, using the AHH assay. I measured the in vitro AHH activity induced by concentrations of coplanar PCBs which approximated those sampled in the field. I dosed cultures with actual sample extracts, and measured the resulting enzyme induction. I also investigated the in vitro enzyme induction produced by mixtures of PCB congeners. Three different cell lines were employed. The highly responsive Hepa Clone9 system was used to investigate the relative potencies of previously unstudied inducers. H4IE cells were used to facilitate comparisons between my results and the literature, and I used the HepG2 cells in order to compare and contrast the human response with that of the mouse and the rat. Additionally, the human cells provide the most medically relevant system with which to assess the hazard and toxic potency of environmental pollutants.
MATERIALS AND METHODS

Cell Lines

The Hepa cl-9 cell line, was derived in the laboratory of Dr. M.J Dufresne, through cloning of the Hepa 1cl cells, which were originally isolated from a C57L/J (B6) murine hepatoma. These cells were maintained in alpha medium supplemented with 5% fetal calf serum, and 50ug/mL of the antibiotic gentamycin. The H4IIE cells were obtained from Reuber hepatoma H-35, and were provided by Dr. E. Brad Thompson, of the National Cancer Institute, Bethesda, MD. The H4IIE rat hepatoma cells were also grown in alpha medium supplemented with 5% fetal calf serum and 50ug/mL gentamycin. The third line of cells employed were the HepG2 human hepatoma cells, which were supplied by Drs. Knowles and Aden of the Wistar Institute of Anatomy and Biology, Philadelphia, PA. This cell line was derived from biopsies of primary liver hepatoblastomas, and was maintained in alpha medium supplemented with 5% 60:40 serum (60% calf serum and 40% fetal calf serum) plus 2mL antibiotic. A shift from 5% 60:40 serum, to 5% fetal calf serum facilitated greater resolution of fluorescent metabolites, and this recipe was subsequently used to maintain the HepG2 cells.

The Hepa cl-9 cells have generally shown the highest levels of AHF induction in response to polycyclic aromatic hydrocarbons (PAHs), and organochlorines (OCs); however, the H4IIE rat hepatoma cell line is the standard bioassay cell line reported in the literature. All three cell lines exhibit similar growth parameters; however, HepG2 tends to grow in a three-dimensional array (cells 'piling' on top of one another), and so takes approximately double the time of Hepa cl-9 and H4IIE to reach confluence (typically 72 -
96h for a 100mm plate initially seeded with 1.0x10^6 cells).

Subculture Technique

The routine subculture of cell lines is essential to their continued survival. Near-confluent, or confluent cells were removed from a 37° incubator and capped tightly if in a flask. In the laminar flow hood (which had been running for at least 0.5h prior to use, and had been cleaned with 70% isopropanol), spent medium was aspirated from the cells with a sterile, unplugged pasteur pipet. The cells were then washed, using a sterile, plugged pasteur pipet, with approximately 2mL citrate saline. The wash solution was removed from the cells (using the same pipet if it had not come in contact with any surfaces under the hood and had been passed through a flame), and the cells were then dispersed from the surface of the culture dish/flask. This dispersion was accomplished by adding 1.0mL of 0.25% or 0.125% trypsin per 100mL plate or 25cm² flask, or 2.0mL trypsin per 80cm² flask, and incubating for 3 - 5 minutes. The cells were removed from the incubator, and checked under the microscope for disassociation. If the cells were free in the medium, the trypsinized cells were aspirated using a plugged pasteur pipet, and were suspended in fresh growth medium in a 100x17mm polypropylene tube. Generally, the trypsinized cells from 80cm² flasks were suspended in 8.0mL medium, to bring the final volume to 10.0mL medium and cells. Trypsinized cells from 100mm plates, and 25cm² flasks were suspended in 4.0mL medium, to give a final volume of 5.0mL medium and cells. If cells were to be seeded for a specific purpose other than to carry the population, a small aliquot of cells was loaded into the
two chambers of a haemacytometer for counting (see Appendix 2). The remaining cells (or all cells if the subculture was for maintenance of the cell line only) were centrifuged at 1000rpm for 6 minutes. While the cells were spinning, the cells in each chamber of the haemacytometer were counted, to determine the number of cells per mL, and the total number of cells. When the centrifuge finished, the supernatant was aspirated from the pellet using a sterile unplugged pasteur pipet, and the pellet was resuspended in an appropriate volume of medium. The concentration of cells per volume of growth medium was determined by the experiment to follow. For AHH purposes, cells were seeded in 100mm plates at concentrations of 5.0x10^6 cells/plate, or 1.0x10^6 cells/plate. Routine subcultures usually entailed adding 2 - 4 drops of resuspended cells to 25cm^2 flasks, and 1.0x10^6 cells to 80cm^2 flasks. The newly seeded culture plates/flasks were then returned to the incubator, after checking the cells under the microscope.

**Aryl Hydrocarbon Hydroxylase Assay**

Cells were grown in 100mm plates until they were 75 to 90% confluent. Hepacl-9 cells were seeded at concentrations of 5x10^5, or 1x10^6, and were grown for 24 to 48h respectively, prior to induction. H4IIE cells were plated at a concentration of 1x10^6, and also approached confluency after 24 to 48h. The HepG2 cells were seeded at 1.0x10^6 cells, and as discussed, took from three to four days to reach confluency. Depending on the number of plates ready to assay, each treatment (drug and control) was replicated 2 - 4 times.

The B[a]P substrate and many of the inducers employed for the AHH assay were
light sensitive or unstable, as well as toxic and/or carcinogenic, thus special care and precaution was taken when preparing or working with these chemicals. PCB congeners IUPAC #77, #126, and #169 were dissolved in acetone at concentrations of 180ug/mL, 140ug/mL, and 150ug/mL respectively. These were then stored in air-tight vials in the fumehood. 2,3,7,8-TCDD was dissolved in DMSO to create a 5x10^{-4}M stock solution, which was stored in the same manner as the PCBs. Experiments were also run with another stock solution of 2,3,7,8-TCDD (1.5x10^{-5}M), generously supplied by Dr. A. Okey of the University of Toronto. Inducers were diluted with solvent, or serially diluted with growth medium in order to reach the desired experimental dosages. The PCB congeners were applied to cells in concentrations which ranged from 0.0001ug/L to 100ug/L. These levels corresponded with the measured residues in species sampled. Dioxin was used as a positive control, and was added to cells in volumes sufficient to create culture environments of 5x10^{-8} or 10^{-9}M (that molarity at which maximal AHH induction occurs). Each AHH assay also employed solvent controls, where solvents were added to cells in 1:1000 dilutions; and two other controls, which underwent reaction lacking either cells or substrate. Induction involved a straight exchange of normal medium for medium containing the chemical (following a citrate saline wash). Cells were then incubated for 18 - 24h if Hepa cl-9 or H411E, and for 30h if HepG2.

Cells were harvested on ice, by decanting the medium (into a PAH/OC waste bottle), and scraping the cells off of the plate with cold phosphate buffered saline (PBS) and a rubber policeman. Cells were then collected into prelabelled polypropylene tubes, and spun down at 1500 RPM for 6 minutes. The supernatant was decanted, pellets were
resuspended in approximately 2mL PBS, and centrifuged again. In total, the cells were washed four times, then briefly inverted over paper towel to drain excess moisture. Cold glycerol phosphate (GPO4) was added to each pellet, in volumes which corresponded to 350uL per plate per tube (i.e. one pellet may have been comprised of 4 separate plates, so 4x350uL GPO4 was added). This volume may be adjusted if the protein yields are low, so as not to dilute the cells, thereby reducing the fluorescence.

Small Erlenmeyer flasks, long stoppered tubes, short stoppered tubes, and 12x75mm disposable tubes, in quantities which corresponded to the number of culture plates harvested, were arranged and numbered prior to, or during the assay. Short stoppered tubes were filled with 3.0mL 1.0N NaOH, and refrigerated until it was time for the extraction in alkali. One disposable tube was also filled with NaOH, in order to blank the fluorometer. The reaction pool was made the day of the assay, and was refrigerated until used. The pool quantity was adjusted through multiplying required weights and volumes by the initial number of seeded plates plus two. Given that the pool contained light sensitive compounds, it was important to work under low light conditions.

On ice, 100uL cells (resuspended in GPO4) were added to 900uL of pool in each small flask. Two control flasks received control cells and no substrate, while two control flasks received pool plus substrate. At this time, it was essential that the lights were turned off, and remained off for the duration of the assay.

50uL of 2mM B[a]P was added to each flask at 15 second intervals, and the flasks were placed in a shaking water bath at 37°C, and 80 shakes per minute. When adding the B[a]P, it was essential to place the pipet tip right into the solution of cells and pool,
in order to ensure a maximal reaction. After 20 minutes, the reaction was stopped through addition of 3mL of cold hexane:acetone (3.25:1 v/v). The flasks were removed in the same order that they had been incubated, capped with marbles (to prevent evaporation), and they were placed on ice. At this time 100uL of cells was added to the control flask which contained pool only, while 50uL of B[a]P was added to the flask containing cells only. The flasks were returned to the water bath for a further 10min to ensure the best extraction of B[a]P and metabolites into the organic layer, then the contents of each flask were poured into the corresponding long stoppered tube.

The long stoppered tubes were vortexed for 5 seconds, and allowed to equilibrate. When two distinct phases were apparent, 1.0mL of the top layer was pipetted off, and added to the chilled NaOH in the corresponding short stoppered tube. The short tubes were vortexed for 25 seconds, thereby extracting phenolic and other polar metabolites into the alkali phase. After equilibration of the short tubes, the bottom phase was pipetted into the corresponding disposable culture tube, and read on a Turner Fluorometer. The samples were extracted in alkali in groups of four, in order to avoid degradation of the fluorescent product. The fluorometer was allowed to warm up for approximately 10min before being turned on. The inside scale was set at 60nm, while the excitation and emission were set at 396 and 522nm respectively. The sensitivity knob was turned as far to the right as possible, the sensitivity switch was set on HIGH, while the blank switch was set to LOW, and the machine was blanked with 1N NaOH. After 'reading' all tubes, the waste was discarded in the PAH/OC bottle, and the cells were stored at 4°C until the protein content was determined. One unit of AHH specific
activity is defined as that amount of enzyme catalyzing the formation of hydroxylated products causing fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo[a]pyrene recrystallized standard per minute at 37°C. Specific activity is expressed in units per milligram of protein and is calculated as follows:

1. Fluorometer readings are 100x, 10x or 3x higher than the actual values, so all fluorometer readings were accordingly multiplied by 10² or 10³ and the mean value for duplicate or triplicate treatments was determined.

2. The mean for each treatment was corrected for protein content of the sample by dividing by the number of milligrams of protein.

3. This value was then corrected for time by dividing by the water bath incubation time of 20 minutes.

4. The resulting number was converted to pmoles 3-hydroxybenzo[a]pyrene by multiplying by 61.44, as 1 fluorometric unit = 61.44 pmoles 3-hydroxybenzo[a]pyrene.

A quinine sulfate standard, with a fluorescence of greater intensity than the maximum expected in the assay, was read before the samples each time an assay was performed. See Appendix B for preparations needed to perform the AH Assay.

Extracts from tissue samples which had been cleaned up and extracted as described in the methods section of Chapter Two were used to induce cells. To facilitate mixing of the medium (a water-based solution) and the extract (hydrophobic compounds), no iso-octane was added to Fraction 2. The absence of this 'keeper' made it necessary to rotocvaporate the extracts very slowly and at low temperatures. The final volume was
reduced to 1mL, and then brought up to 5mL using acetone, as a more water-soluble and miscible solvent. Extracts were stored in airtight vials and refrigerated until used.

**Haemacytometer Use**

To determine the total cell number and the number of cells per mL, the centre square in each chamber of the haemacytometer was counted. If the number was equal to or greater than 50 it was multiplied by $10^4$ to obtain cells/mL. If the number of cells in the centre squares of the haemacytometer did not exceed 50, it was necessary to count the cells in each of the four corners, add this to the centre number and then multiply by $2.0 \times 10^3$ to determine cell/mL. The total number of cells was then determined by multiplying the total volume (trypsinized cells plus fresh growth medium) by the number of cells/mL.

**Cloning**

Cells were trypsinized from one 100mm plate as already detailed and counted. Cells were resuspended in an appropriate volume which facilitated serial dilutions that resulted in final concentrations of 10 and 5 cells per mL. Four drops of each concentration were added to one 96-well Linbro cloning tray, and a slightly higher concentration was used for 'focus wells'. In order to minimize contamination the tray was only checked periodically. After one week several clones (wells which contained only one colony) were selected and transferred into individual wells in a larger Linbro tray (perhaps 24 wells) which contained fresh growth medium. The subculture technique
was modified to accommodate such small numbers of cells by reducing the volume of trypsin to two drops per well.

Hepa cells were cloned in order to select for the highest inducer when AHH activity levels had dropped to background levels.

**Efficiency of Plating**

In order to establish the efficacy of the stock B[a]P, cells were seeded in varying concentrations of this drug. After one week plates were washed with citrate saline and stained with methylene blue for 10 minutes. The stain was then poured off and the plates were rinsed with tap water. After drying, each plate bottom was divided into quarters and the number of colonies per quarter was determined. E.O.P. revealed that the B[a]P was efficacious as increasing B[a]P molarities had killed increasing numbers of colonies.
RESULTS

Coplanar PCBs do induce AHH. The highest induction was seen in Hepa Clone9 cells during the summer of 1991 (Figure 4.3). There were significant differences among treatments for both the August 7 and August 24 assays ($P=0.023$, $H=30.19$, 17DF, $P=0.025$; $H=40.05$, 21DF, $P=0.007$) (all statistical analyses excluded zero controls). Congeners #126 (at 2.9x10-2 M) and #169 (at 3.2x10-2 and 6.4x10-3 M) stimulated activity which was comparable to that of 2,3,7,8-TCDD (at 2.5x10-8 M). In contrast, PCB #77 at all treatment concentrations induced much lower levels of AHH. Combinations of coplanars gave inconsistent results. Mixtures of congeners in the August 7 assay produced background levels of induction, whereas applying congener mixtures to cells for the August 24 assay resulted in high levels of induction.

Later assays resulted in much lower enzyme induction despite similar PCB treatments and molarities (Figure 4.4). While there were no significant differences found among treatments for either the February 5 or February 12 assays ($H=8.34$, 9DF, $P=0.5$; $H=9.34$, 10DF, $P=0.5$) it does appear that the level of enzyme activity increases with increasing congener molarity. There were significant differences in the AHH activity among treatments for the May 28 assay ($H=60.02$, 6DF, $P<0.001$). Consistent with earlier results, PCB #126 induced more strongly than did congeners #77 and #169.

Hepa Clone9 was more inducible than either H4IIIE or HepG2 (Figure 4.5). There were significant differences in enzyme response to treatment with PCB #77.
between H4IIE and Hepa Clone9 (F = 10.33, 2DF, P = 0.022). The Hepa cells responded more strongly to all molarities of PCB #77 than did the H4IIE cells. No significant differences were found between HepG2 and Hepa Clone9 (F = 5.88, 1DF, P = 0.134); however, assays performed using only 2,3,7,8-TCDD, revealed much higher levels of AHH activity in HepG2 than suggested by the May 11 results.

The overall levels of AHH activity fell for all cell lines, as can be seen in Figure 4.6. PCB #126 which maximally induced Hepa cells six months earlier (25 fluorometric units), generated AHH levels that were not significantly greater than acetone and control treatments (0.13 units of specific activity, H = 1.59, 7DF, P = 0.995). TCDD also exhibited reduced inducing potential. No significant differences were found among the contaminant treatments used during the April 29 assay (H = 7.519, 5DF, P = 0.5); however, TCDD appears to have induced higher levels of AHH than did all molarities of #126, which produced similar levels of enzyme activity.

PCB #77 exhibited a general trend in Hepa cells of higher AHH induction with increasing molarity (Figure 4.7). There were no significant differences among treatments for the November 2 assay (H = 7.34, 8DF, P = 0.5) while there were significant differences among treatments for the November 6 and January 27 assays (H = 16.91, 9DF, P = 0.05; H = 14.06, 7DF, P = 0.05). Once again, the levels of induction produced in these assays are much smaller than those recorded for the August assays. PCB #77 induced enzyme activity at molarities of 2.9x10^{-2} and 5.8x10^{-3} which were comparable to those levels induced by TCDD (10^{-7} and
10^{-9} \text{ M}).

Figure 4.8 shows the results of two assays using extracts that were obtained from cleaned-up fish and gull egg tissue. The extracts induced minimally, and did not differ in AHH induction ($H = 12.01$, $7 \text{DF}$, $P = 0.1$). Figure 4.9 details more extract results. The upper and lower graphs represent the same data; however, the levels of enzyme activity for TCDD and PCB #126 (1.3x10^{-3} \text{ M}) were so high they obscured the other treatment results. Once again, the extracts induced little AHH activity.
The AHH activity expressed as specific activity (units of enzyme/mg protein), for Hepa Clone 9 cells exposed to nothing (C), acetone (ACE), TCDD, PCB #77, PCB #126 and PCB #169 at four different molarities. Cells were also exposed to PCB combinations at two different molarities. TCDD was used at 2.5x10^-8 M. Error bars represent ±1 standard error. BLNK refers to NaOH used to zero the fluorometer. The assay date is recorded in the upper right corner of each bar graph.
Specific Activity (enzyme units/mg pr)

07/08/91

24/08/91

Molarity (moles/L) and Treatment

X=1.2x10^-3

77, 125, 169, 24x10^-4

77+125+169, 3.5x10^-4

8.8x10^-2

1.2x10^-3

77, 125, 169, 1.2x10^-3

1.3x10^-3
The AHH activity expressed as specific activity (enzyme units/mg protein) for Hepa Clone9 cells exposed to nothing (C), acetone (ACE), TCDD, benzene (BENZ), PCBs #77, #126 and #169 at various molarities. Error bars represent ±1 standard error. The assay date is recorded in the upper right corner of each bar graph.
Figure 4.5  The AHH activity expressed as specific activity (enzyme units/mg protein) for Hepa Clone9, H4IIIE and HepG2 cells, exposed to nothing (C), acetone (ACE), benzene (BENZ), TCDD, and various molarities of PCBs #77, #126 and #169. Error bars represent ± 1 standard error. The assay date is in the upper right corner of each graph.
The AHH activity expressed as specific activity (enzyme units/mg protein) for Hepa Clone 9 cells exposed to nothing (C), acetone (ACE), benzene (BENZ), TCDD and five molarities of PCB #126. Error bars represent ± 1 standard error. The assay date is in the upper right corner of each bar graph.
Figure 4.7 The AHH activity expressed as specific activity (enzyme units/mg protein) for Hepa Clone9 cells exposed to nothing (C), acetone (ACE), benzene (BENZ), TCDD, and various molarities of PCB #77. Error bars represent ± 1 standard error. The assay date is in the upper right corner of each bar graph.
Figure 4.8  The Ahh activity expressed as specific activity (enzyme units/mg protein) for Hepa Clone9 cells exposed to nothing (C), acetone (ACE), TCDD, PCBs #77 and #126, and various concentrations of extracts from gull eggs and smallmouth bass muscle. Error bars represent ± 1 standard error. BLNK refers to a NaOH sample used to zero the fluorometer. Assay dates are recorded in the upper right corner of each bar graph.
Concentration, Molarity and Treatment
Figure 4.9  The AH activity expressed as specific activity (enzyme units per mg/protein) for Hepa Clone9 cells exposed to various molarities of PCB #77 and PCB #126, as well as to several different concentrations of gull egg and smallmouth bass muscle sample extracts. The lower graph represents the data from the upper, without the outlier TCDD and PCB #126 at 1.3x10-3 M. Error bars represent ± 1 standard error. The assay dates are located in the upper right corner of each graph.
Concentration, Molarity and Treatment
Discussion

The AHH assay is a potentially sensitive indicator of low level, chronic exposure to pollution. This was the initial attempt by our laboratory to learn the assay procedure, to establish standard dose-response curves for the coplanar congeners, and to develop a protocol for applying sample extracts to cells. The AHH assay exhibited promise as a powerful method for elucidating congener-specific activity, as well as for gaining insight into congener and multiple chemical interactions. The technique needs to be consistent, however, before it can be used as a dependable monitoring tool.

Assay results were temporally variable, and I had difficulty obtaining quantifiable results from November 1991 through June 1992. I altered every aspect of the assay (solutions, chemicals, cells, scraping techniques, lab procedures and incubation time) in an attempt to rectify the problem; however, induction levels remained low. I initially used Hepa cl-9 cells only for the PCB-dosing assays. These cells are highly inducible and therefore ideal for revealing small differences in the inducing potency of different congeners. When levels of induced AHH fell to background values in the mouse cell line, I turned to the less responsive but more relevant H4IIE and HepG2 cells. My initial plan had been to establish induction dose-response curves for each PCB congener using the Hepa cl-9 cell line, and then compare the murine system with the rat and human systems. The atypical and inconsistent responses by my Hepa cells necessitated switching to the rat and human cells prematurely. The problem is apparent in Figure 4.5, where Hepa cl-9 cells
express levels of AHH activity which are comparable to or lower than levels in human and rat cells.

Despite the considerable variability in assay results, there were some consistent observations. PCB #126 maximally induced Hepa cl-9 cells relative to the other non-ortho substituted congeners. Cells exposed to #126 at molarities of $10^3$ and $10^3$ induced AHH levels similar to those produced by cells exposed to $10^{-7}$ to $10^{-9}$ moles/L dioxin. This supports toxic equivalents research that indicates coplanar PCBs represent greater hazard to wildlife than dioxin because they are more abundant in the environment.

Mixtures of congeners did not act additively. Combinations of #77, #126 and #169 induced lower levels of AHH than comparable molarities of individual congeners. Similarly, cells exposed to extracts exhibited low levels of induction. I was unable to determine whether this was due to chemical competition for Ah receptor binding sites or was a result of insufficient cell exposure. Current research using the HepG2 system indicates that PCB #77 competitively inhibits Ah receptor binding (and AHH induction) by TCDD when the two are simultaneously applied to cell cultures (Dr. Dufresne pers. comm.). Congener #77 is a smaller, more soluble organochlorine which is postulated to cross cellular membranes faster than 2,3,7,8-TCDD. If #77 were to occupy the Ah receptor binding site and prevent TCDD from binding, this would result in much lower levels of induced cytochrome P450. The extracts I applied to cell cultures contained many structurally similar compounds. It is possible that a highly soluble organochlorine with low potency competitively
inhibited Ah receptor binding by high inducers, and this caused the low levels of enzyme activity which I observed.

Another explanation for the low levels of AHH activity induced by extracts is that the cells were not sufficiently exposed to the inducing compounds because of phase separation. It was difficult to apply cleaned-up tissue extracts to cell cultures because of the non-polar nature of the extracts. Cells dosed with tissue extracts were slippery and greasy when being scraped, and often I could see the extract floating as a separate phase on top of the medium. I removed iso-octane (2,4,5-trimethylpentane) from the second fraction clean-up, and resuspended the extract in acetone instead of hexane. The results following these changes were not significantly different. Studies by Casterline et al. (1983) and Ankley et al. (1991) also used the AHH assay to measure residue levels of P450 inducers in extracts from fish flesh. I found the techniques used by these researchers to be ineffective. Both Casterline and Ankley resuspended their extracts in isooctane and then added this to the cell cultures. Isooctane is not miscible in alpha medium and it is unlikely that hydrophobic contaminants such as PCBs and dioxins would partition into the polar alpha medium which feeds the cells. I suggest that the cells were not sufficiently exposed to inducers. The Casterline and Ankley papers also adopted unusual dosing procedures as they incubated H4IIIE cells with extracts and TCDD for 72 hours. According to the literature Hepa cl-9 and H4IIIE exhibit maximal enzyme induction following 18-24 hours of xenobiotic exposure (Okey et al. 1980). The activity values reported by these authors may be experimental artifacts given the lengthy exposure time.
Additionally the authors included a TCDD dose-response curve which indicates that TCDD maximally induces AhH activity at a molarity of $5 \times 10^9$ in contrast to the literature value of $1.0 \times 10^9$ (Okey et al. 1980).

A final explanation for the low induction seen when cell were exposed to multiple inducers is the incubation time. No time-course studies using PCB congeners were performed to determine when cells were maximally induced. Low levels of induction during multiple-chemical dosing may be correlated with a reduced capacity to express AhH with exposure time. This phenomenon has been recorded in chronically exposed fish which exhibit a reduced capacity to respond to additional contaminant inputs (Monoson et al. 1991). Given the rapid growth and generation time in vitro, cells may be in a state of chronic exposure after 24 hours incubation with PCB inducers and perhaps should be assayed earlier in order to measure maximal enzyme activity. Monoson et al. (1991) also found that greater doses of PCB #77 (10 mg/kg) decreased the catalytic efficiency of P450IA1 enzyme. This dosing concentration is 100 fold larger than my highest molarity of #77. However, my sample extracts were very contaminated and it is possible that they contained concentrations of inducers in the parts per million range. If this were true, once again some form of chemical inhibition could be taking place at the level of the Ah receptor.

AhH has been widely studied. However, some papers suggest that 7-Ethoxyresorufin-O-dealkylase (EROD) is a superior enzyme to monitor because it is more specifically induced by coplanar xenobiotics (Andersson et al. 1988; Melancon
cii al. 1987). It would be beneficial to investigate the induction of this MFO as an alternative and perhaps more consistent biomonitoring assay, although EROD is not well understood and there is little knowledge about the mechanism of EROD induction. Aryl Hydrocarbon Hydroxylase is such a powerful assay because a direct relationship has been established between xenobiotic binding to the Ah receptor and AHH activity.

I recommend that a similar study be carried out which considers all of the assumptions necessary for parametric testing. A three-level nested anova assessing differences among cell lines and treatments seems an ideal approach to discovering significantly different levels of induction.

It is essential, given the widespread environmental contamination by PCBs and other contaminants, that an understanding of chemical interactions and effects is reached. The AHH or EROD assays may be the approaches needed to determine the toxicological hazards associated with exposure to multiple xenobiotics. Through the AHH assay it may be possible to discover antagonists which could alter xenobiotic toxicity through binding interference. Ideally this assay should be used as a screen to measure the 'total biological activity' of complex environmental samples. This information would make the data in a chromatogram more meaningful in an ecological and biological sense.
CHAPTER FIVE
GENERAL DISCUSSION

The 1993 Guide to Eating Ontario Sport Fish is now available to consumers in liquor stores across the province. Noticing this publication propped next to the bottle bags at the check-out counter reinforced what my two years of contaminant study had demonstrated: polychlorinated biphenyls are a fact of life. These contaminants are abundant in the global environment and restrictions on Great Lakes fish consumption are only one small result of PCB pollution.

My research revealed that PCBs are present in significant quantities in Lake Erie sediment and biota despite fifteen years of restricted production and use. The 209 PCB congeners do not exhibit similar distributions among biotic and abiotic compartments because they partition differently due to selective biodegradation, biotransformation and bioaccumulation. Total PCB does not adequately describe sample residues. Additionally such a measure neglects to document valuable modelling information.

Coplanar (non-ortho) PCBs also partition and enrich differently than do dominant congeners, such as #138 and #153, which are considered to be representative of total PCB. While sample concentrations of the coplanars were small relative to other congeners, the levels were still large enough to pose a significant toxic threat to fish, wildlife and humans. Despite claims to the contrary (Williams et al. 1992) the concentration of total PCB in samples did not adequately predict TCDD-equivalents. My research revealed that total PCB concentration increased with increasing trophic level,
but the percent of total PCB represented by the coplanars decreased.

AHH activity proved to be a sensitive but problematic exposure indicator. I believe that this assay could be an invaluable tool for the development of QSARs and for elucidation of contaminant interactions in vivo. I strongly recommend continued research using cell culture and AHH, and suggest that an individual with a strong molecular background and AHH as their sole focus could 'work out the bugs' in this system. My research did reveal interesting trends including the relative induction potencies of the three coplanar congeners (#126 > #169 > #77).

Looking over my data and conclusions it is apparent that a logical (and important) extension of this study is to expand. A more comprehensive data base needs to be compiled; one that includes large volume water samples, zebra mussels, forage fish, and adult gulls. All samples need to be well-replicated (n = 10) so that parametric analyses may be effectively used. Additionally, because the exposure history of sampled fish and wildlife is not known, large sample numbers will help to identify outlier values. Tissue-specific analysis for fish and birds should continue in order to gain some understanding of contaminant dynamics within organisms.

Exposure data becomes more meaningful when paired with biological effects data. The successful application of sample extracts to cell cultures would facilitate the identification of hazardous combinations and concentrations of congeners. My research was an initial attempt to combine the ecology of contaminant dynamics in the ecosystem with the dynamics and effects of contaminants at the molecular level. Given that PCBs act on individuals to produce ecosystem-wide effects it is essential that these two
disciplines (ecotoxicology and toxicology) be married.

The future of PCB contamination will be long. It is important that researchers begin now to map the distribution and exposure dynamics of these ubiquitous organochlorines. It is equally important that emphasis be placed upon physiological and epidemiological studies so that levels of PCBs in the environment can be used to predict biological effects.
Literature Cited


130


Norstrom, R.J. Bioaccumulation of polychlorinated biphenyls in Canadian wildlife. Summary report for Environment Canada, C.W.S., National Wildlife Research Centre, Ottawa, Ontario, K1A 0H3


APPENDIX B: Assay Preparations

Assay pool

dH₂O (distilled water)  0.62mL per plate
0.1M MgCl₂  0.03mL  "
0.2M Tris Buffer pH 7.5  0.25mL  "
NADH  0.3mg  "
NADPH  0.3mg  "
Bovine Serum Albumin  0.7mg  "

Store at 4°C until added to flasks.

Benzo(a)Pyrene [MW = 203.3]

Given that it is undesirable to store large quantities of this known carcinogen, which is also subject to considerable degradation over time, the initial B(a)P recipe was reduced, creating a smaller stock solution. 10mgs B(a)P is dissolved in 0.4mL benzene, and then the volume is adjusted to 19.8mL using methanol. This procedure must be carried out with the overhead lights off.

Glycerol Phosphate Buffer

K₃HPO₄  44g
KH₂PO₄  9.2g

glycerol  300mL (warmed in water bath)

Dissolve the first two compounds in the warm glycerol, then adjust the volume to
1000mL with dH₂O. Ensure the pH is 7.5, and store at 4°C until needed.

II. Hexane:Acetone

Mix analytical grade hexane and acetone in a 3.25:1 ratio, store at 4°C.

0.1M Magnesium Chloride [MW = 95.23]

MgCl₂  9.523g/ldH₂O, store at 4°C.

1.0N NaOH [MW = 40]

NaOH  40g/ldH₂O, store at 4°C.

Phosphate Buffered Saline

NaCL  8.0g/l
KCL  0.2g/l
Na₂HPO₄  1.14g/l
KH₂PO₄  0.2g/l, store at 4°C.

Quinine Sulfate

Prepare a quinine sulfate working solution (2ug/mL in 0.1N H₂SO₄), and then prepare the following dilution series:
<table>
<thead>
<tr>
<th>Final Conc. (µg/mL)</th>
<th>Quinine Sulfate vol.</th>
<th>0.1N H₂SO₄ vol.</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>2mL of 2µg/mL</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>1.0</td>
<td>2mL of 2µg/mL</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>0.5</td>
<td>2mL of 1µg/mL</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0.25</td>
<td>2mL of 0.5µg/mL</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>0.125</td>
<td>2mL of 0.25µg/mL</td>
<td>2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Read these concentrations directly in the Turner fluorometer. If the standard curve does not give a close approximation of the above fluorescence readings, the following measures may be necessary:

1. Check the quinine sulfate solution - it is possible to store the quinine sulfate standards at 4°C in the dark for several weeks; however, sometimes the solution appears to degrade faster than expected.

2. Realign the bulb, and perform the standardization suggested in the fluorometer manual - this requires the assistance of the equipment technician.

3. Replace the bulb.

4. Redo the quinine sulfate to 3-OH B(a)P curves.

0.2M Tris buffer, pH 7.25  [MW = 121.1]

24.23g Tris/1dH₂O, store at 4°C.
VITA AUCTORIS

NAME: Susan Elizabeth Koslowski

PLACE OF BIRTH: Hamilton, Ontario

YEAR OF BIRTH: 1967


